POLYMETHACRYLATE PLATFORMS WITH CONTROLLABLE SURFACE PROPERTIES FOR DENGUE VIRUS DETECTION

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Abstract

This thesis presents an effective method for synthesis and processing of polymethylmethacrylate-co-methacrylic acid (PMMA-co-MAA) biochips with controlled surface properties aimed for successful detection of the Dengue virus (DV). By variation of synthesis reaction parameters and using spin-coating technique, different compositions of the PMMA-co-MAA coated biochips were prepared. Designed coated chips have been employed for surface immobilization of the Dengue antibody molecules and further detection of DV through antibody/antigen coupling. Biomolecule immobilization on the functionalized surfaces of the chips was performed via different techniques including: 1) physical adsorption of the antibody on the surface; 2) covalent immobilization of the antibody on the surface through carbodiimide chemistry; and 3) antibody immobilization via different sizes of the amine-bearing surface spacers, hexamethylenediamine (HMDA) and polyethyleneimine (PEI). Results have shown a higher detection signals generated from polymer coated biochips in comparison to the conventional enzyme-linked immunosorbent assay (ELISA). Particularly, the presence of amine spacers on the surface enhanced the detection signal up to the considerable levels (~ 8 times higher signal intensity than conventional method). Developed biochips in this study are privileged with strong and permanent presence of surface carboxyl (–COOH) groups that can be essential in successful protein immobilization and subsequent detection of the DV. Presented biochips have shown a great potential for future integration into the biodiagnostic tools such as microfluidic devices. Such protein-friendly platforms can be employed for detection of different types of viruses not just limited to DV.

Abstrak

Tesis ini menerangkan tentang kaedah yang lebih berkesan untuk sintesis dan pemprosesan metil asid poli metacrylate-bersama metacrylic PMMA-bersama MAA biochips dengan ciri-ciri permukaan yang dikawal dan bertujuan untuk mengesan virus Denggi yang (DV). Dengan memvariasikan parameter tindak balas sintesis dan menggunakan teknik spin-lapisan, komposisi berbeza daripada (PMMA-co-MAA) bersalut biochips telah disediakan. Rekaan chip bersalut tersebut telah digunakan untuk imobilisasi antibodi Denggi dan mengesan lebih lanjut virus Denggi (DV) melalui gandingan antibodi/antigen. Imobilisasi biomolekul satu permukaan befungsikan chip telah dilakukan melalui teknik yang berbeza termasuk: 1) penyerapan fizikal antibodi pada permukaan; 2) imobilisasi kovalen antibodi pada permukaan melalui kimia karbodiimida dan 3) imobilisasi antibodi dengan saiz yang berbeza dari spacer permukaan amina-bearing, heksametilenadiamina (HMDA) dan polyethyleneimine (PEI). Keputusan telah menunjukkan isyarat pengesanan yang jauh lebih tinggi dijana daripada cip polimer dibandingkan dengan teknik konvensional esei imunoserapan (ELISA) assay enzim-linked. Terutamanya, kehadiran spacer amina di permukaan telah meningkatkan isyarat pengesanan sehingga ke tahap yang cukup besar (keamatan isyarat ~ 8 kali ganda lebih tinggi daripada kaedah konvensional). Biochips yang dimajukan di dalam kajian ini adalah istimewa kerana permukaannya mempunyai kumpulan karboksil (–COOH) pada kedudukan yang kukuh dan kekal yang penting dalam pergerakan protein yang menjadi kejayaan untuk pengesanan DV. Biochips yang dihasilkan di dalam kajian ini telah menunjukkan potensi yang besar untuk diintegrasikan ke dalam alat-alat yang biodiagnostik seperti peranti microfluidic pada masa hadapan. Platform mesra protein seperti ini boleh digunakan untuk mengesan pelbagai jenis virus dan tidak hanya terhad kepada DV.

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Table of Contents

List of Figures and Tables

List of abbreviations

AFM	Atomic force microscopy
AIBN	Azobisisobutyronitrile
BSA	Bovine serum albumin
DAP	1,2-diaminopropane
DV	Dengue virus
DF	Dengue fever
DHF	Dengue hemorrhagic fever
DSS	Dengue shock syndrome
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
ELISA	Enzyme-linked immunosorbent assay
FN	False negative
FP	False positive
GA	Glutaraldehyde
HMDA	Hexamethylenediamine
LoD	Limit of detection
MAA	Methacrylic acid
MMA	Methyl methacrylate
Na_2HPO_4	Disodium hydrogen phosphate
NaH_2PO_4	Monosodium phosphate
NHS	N-hydroxysuccinimide
PBS	Phosphate buffer saline
PEI	Polyethylenimine
PMMA-co-MAA	Poly methylmethacrylate-co-methacrylic acid,
SEM	Scanning electron microscopy

TB	Toluidine blue
THF	Tetrahydrofuran
TN	True negative
TP	True positives
WCA	Water-in-air contact angle
WHO	World health organization
XPS	X-ray photoelectron spectroscopy

CHAPTER 1: Introduction

1.1 Problem statement

One of the most commonly applied diagnostic tools in detection of viruses is enzyme-linked immunosorbent assay (ELISA). ELISA is also known as immunoassay method in variety of applications such as determination of food allergens, concentrations of blood serum antibody, concentrations of particular types of drugs and detection of cancer biomarkers (Kirsch, et al., 2013; Lin, Kekuda, & Chu, 2010). Despite variety of applications of this analytical method, ELISA has shown many serious drawbacks such as time consuming and tedious procedure, inconsistency of the results, relatively low limits of detection and high sample volume required for detection (Xu et al., 2006). Perhaps one of the major drawbacks of this widely applied method that has been relatively ignored is the performance of polymer surfaces (polystyrene (PS) and polymethylmetacrylate (PMMA) well-plates) as ELISA substrates. Being chemically inert polymers, PS and/or PMMA do not contain desirable reactive functional groups such as amine ($–NH_2$), carboxyl (–COOH) or hydroxyl (–OH) groups, therefore as supporting substrates, do not specifically promote adsorption of proteins. In particular, the sensitivity of analytical kits is of the key importance for early diagnostics and successful treatment of patients (Hosseini, et al., 2014c; Lai et al., 2004). In some cases, the early diagnosis of infection is considered as the most important factor for successful treatment of infected patients and better surveillance (Liu et al., 2012). In that perspective, there is a clear need for well-designed polymeric platforms with desirable surface functionality in the optimum concentration and high level of control over surface properties that can preserve the biomolecule activity and lead to the higher detection sensitivity.

1.2 Recent advances

In order to overcome drawbacks of conventional ELISA, researchers have focused on the development of functionalized polymer platforms with high degree of control over surface properties (Audouin et al., 2012; Bucatariu et al., 2013). In that perspective, poly(acrylate) and poly(methacrylate) macromolecules have drawn a great deal of interest due to their unique properties such as low specific weight, high impact resistance, transparency and flexibility (Bai et al., 2006; Hosseini et al., 2014). Many diagnostic devices depend on the careful surface design of the surface functional groups on polymeric platforms (Audouin et al., 2012; Bucatariu et al., 2013; Yonamine et al., 2012). The modern biosensors to a large extent rely on heterogeneous processes such as immobilization of antibodies/antigens on the bioreceptor surface and measurement of the generated signal through specific coupling between antibody and antigen (Jung, Jeong, & Chung, 2008). For that reason, the production of surface engineered platforms that can act as biomolecule-friendly environments presents the most important aspect in the development of efficient biosensors (Hosseini et al., 2014a; Mitchell, 2011).

A great number of research works report the generation of –COOH functional groups on PMMA plastic by surface modification techniques such as wet chemical (Bai et al., 2006; Brown et al., 2006; Varma, Sreenivasan, Yokogawa, & Hosumi, 2003), UV radiation (Situma et al., 2005) and plasma treatment (Coad, et al., 2013; Li, Y. Ma, S. Wang, & Moran, 2005; Tennico et al., 2010). Those methods might be effective in reported cases but there are still some major concerns about such treatments. Apart from plasma treatment, which offers great deal of control over surface properties, wet chemistry treatments and UV treatment make insignificant changes on the surface. In such cases, “functionalized surfaces” last for a very brief period of time (aging effect) in which the surface energy goes back to a minimum level by means of “surface re-organization” (Vesel & Mozetic, 2012; Hosseini et al., 2014). Therefore, the major

requirement for fabrication of qualified bioreceptor surfaces is to create the functionalized platform with the permanent existence of surface functional groups that would not be affected by aging phenomenon and can be confidently used for biomolecular recognition.

1.3 Proposed strategy

Instead of applying relatively uncertain surface modification methods for generation of desirable functional groups on PMMA polymer, fabrication of biochips coated with synthesized copolymer by using methylmethacrylate (MMA) and methacrylic acid (MAA) monomers in different concentrations of monomers (Figure 1.1) was proposed.

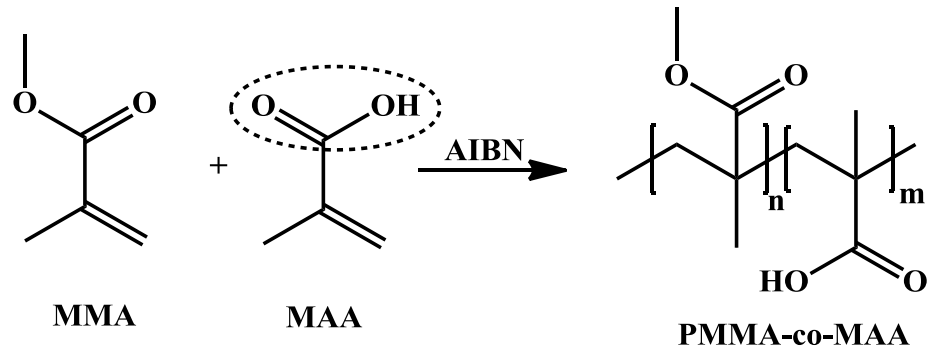


Figure 1.1: Proposed synthesis and chemical structure of PMMA-co-MAA.

With this approach there should be no concern regarding the stability of the inherited –COOH groups at the outermost surface layers of polymethylmethacrylate-co-methacrylic acid (PMMA-co-MAA) copolymers (Hosseini et al., 2014a). By preparation of different compositions of the copolymer in different molar ratios of the monomers (MMA/MAA), a varied range of –COOH concentration is expected to be achieved. This particular feature can be effectively used for protein immobilization. By using spin-coating technique copolymers of different compositions have been processed for preparation of the biochips of 4 mm × 4 mm that have a suitable dimension for integration into the ELISA well-plate used for Dengue virus (DV) detection.

DV belongs to the family of *Flaviviridae,* which can cause the mosquito-borne viral infection, Dengue fever (DF). According to the estimation of the world health organization (WHO), the number of the DV infections exceeds to 50 million annually (Alcon et al., 2002; Stevens et al., 2009). Self-limiting febrile disease, DF, can be associated with life-threatening manifestations such as Dengue hemorrhagic fever (DHF) and Dengue shock syndrome (DSS), which are often ascribed to 1-5% and 10-30% of the recorded cases, resulted in death (Xu et al., 2006).

Functional polymeric surfaces were used to achieve immobilization of Dengue antibody and subsequent detection of DV by three different techniques as follows: (1) physical attachment of biomolecule to the surface of the chips; (2) covalent attachment via carbodiimide chemistry and (3) covalent immobilization by introducing surface amine spacers of two sizes. Spacers are the molecules with available functional groups that can act as intermediate compounds, facilitating the attachment of the proteins to the surface with higher spatial freedom. Those amine spacers were covalently bonded with PMMA-co-MAA surfaces of biochips by means of carbodiimide chemistry.

1.4 Objective of study

The objective of this research study is to develop polymethacrylate platforms with controlled surface properties that can be effectively used for detection of DV.

In order to achieve this objective, the sub-objectives have been defined as follows:

- To develop the polymeric platforms with controlled surface concentration of functional groups and detailed investigation of material properties with major focus on surface chemistry and morphology.
- To confirm the permanent existence of surface functionalities on the biochips that would not be affected by aging phenomenon and can be confidently used for biomolecule immobilization and successful virus detection.

- To establish relationship between tunable chemical structure and surface properties.
- To immobilize the Dengue antibody molecules on the biochips via surface –COOH groups and to obtain enhanced detection signal via different immobilization techniques.
- To study the effect of different immobilization methods on the detection performance of the developed platforms.

1.5 Thesis organization

A complete literature review on available synthetic polymers used as biosensors, different functionalization techniques, their advantages and disadvantages and variety of immobilization techniques are presented in *chapter 2*.

Chapter 3 describes the synthesis of different PMMA-co-MAA compositions, processing of the copolymers for the successful preparation of the biochips by using spin-coating method and different characterization techniques that have been employed for detailed analysis of the polymer structure, surface chemistry and morphology of the coatings.

C*hapter 4* presents further analysis of PMMA-co-MAA in a greater detail. The polymer compositions have been analyzed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-ToF-MS). In this chapter the presence of pendant –COOH groups and structure of the polymer chains of different compositions have been carefully studied. Furthermore, given information about end-groups of the detected chains has provided valuable information about different compositions of PMMA-co-MAA and their application in biosensor domain.

Integration of biochips inside the ELISA analytical kit and successful enhancement of DV detection via physical and covalent attachment are described in *chapter 5*. This

chapter also explains possible re-orientation of the surface functional groups on the biochips of up to 9 months old. In that sense, *Chapter* 5 addresses the major concern regarding the aging effect and stability of the generated surface functional groups in the period of up to 9 months.

Chapter 6 describes the effect of amine spacers on the performance of the biochips aimed for DV detection. The role of two different sizes of the amine spacers and the influence of such linkers on detection performance are discussed in this chapter.

Chapters 2 to *6* present published articles listed at the end of the thesis (list of a publications), which includes front pages of each manuscript, respectively. *Chapters 3* to *6* are experimental articles that contain a brief introduction and literature review. Moreover, mentioned chapters include methodologies related to the published article.

The thesis concludes in c*hapter 7* with some recommendations and suggestions for future directions in this research work.

CHAPTER 2: LITERATURE REVIEW

Recent advances in surface functionalization techniques on polymethacrylate materials for optical biosensor applications

2.1 General introduction

Biosensors, "biochip" or "lab-on-chip" devices are important in various fields such as food safety, medical diagnostics, environmental regulation, and many others (Cheek et al., 2001; Chin, Linder, & Sia, 2007; Crowley & Pizziconi, 2005; Grow et al., 2003; Liu et al., 2004; Novak et al., 2007; Paweletz et al., 2000; Rosty et al., 2002; Vo-Dinh et al., 1999; Wensink et al., 2005a; Xiao et al., 2001). The major principle behind such devices is that the signal is generated by the recognition of an analyte by a bioactive compound and the generated signal is further analyzed by different transduction methods such as: electrochemical biosensors, fluorescence-based detection, cyclic voltammetry, electrochemical impedance spectroscopy, surface plasmon resonance (SPR), potentiometric signal transduction, gravimetric, thermal and other analytical techniques (Kirsch et al., 2013; Le et al., 2011; Liu et al., 2012; Turner, 2013). Recent advances in biosensor technology also include quartz crystal microbalance (QCM) (Boujday et al., 2008) and Fourier transform infra-red attenuated reflection (FTIR-ATR) based sensors that allow determination of spectroscopic signatures of the interacting molecules (Gosselin et al., 2012). In most cases bioactive compounds are proteins such as antigens, antibodies, DNA, cell-binding proteins, etc. One of the major challenges is preservation of protein activity in order to achieve high sensitivity and accuracy of biosensors (Jung et al., 2008). In the view of current analytical challenges, smaller molecules called "aptamers" have been used in order to overcome the limitations of conventional antigens/antibodies (Boujday et al., 2008). The use of smaller molecules such as protein sequences, can possibly

offer better stability and less complicated analytical procedures. Whichever the approach is used, there is still a strong demand for high accuracy, selectivity, lower detection limits and robustness of biosensors (Liu et al., 2012).

2.2 Synthetic polymers as platform for biosensing applications

In recent years, polymeric materials have played a vital role in fabrication of bio-diagnostic devices. Such substrates have shown a high potential in sensitivity improvement and ultimately the miniaturization of bio-analytical devices (Liu & Li, 2012). The ideal biosensor should possess high sensitivity, high accuracy (detectability) and selectivity, cost effectiveness of manufacture protocol and mobility (Lin et al., 2010). One of the widely known applications of such polymer-based devices is a technique called enzyme linked immunesorbent assay (ELISA) (Liu et al., 2009). ELISA is currently used in many applications such determination of serum antibody concentrations, food allergens, concentrations of certain types of drugs, as well as detection of cytokines and cancer biomarkers (Kirsch et al., 2013). Possibly the most well-known clinical application of ELISA tests world-wide is the detection of HIV virus infection in human blood (Kirsch et al., 2013). The basic principle of this method relies on antibody (protein) immobilization on polymeric surfaces and subsequent antigen detection in the blood samples from infected humans. One of the most significant challenges of manufacturing viable biosensors devices is the correct choice of solid surface and the development of appropriate surface chemistry, using a diverse range of proteins: proteins immobilized on the solid surface must maintain their integrity, native conformation, and biological function/activity (Liu et al., 2009). Protein attachment must be closely controlled with respect to chemical selectivity; the functional groups of proteins that are directly immobilized to the functional groups of the polymer surface must be defined in detail (Jonkheijm et al., 2008).

There are many parameters that would influence protein activity when placed in direct contact with the surface. For that reason it is desirable to control regio-selectivity of reaction (protein immobilization) as the protein orientation could vary thus compromising the effectiveness of immobilization methods (Goddard & Hotchkiss, 2007). Possibly the most common surface functionalization of polymers is depicted in Fig 2.1: the first step in successful protein immobilization is the formation of chemical functionalities such as hydroxyl (–OH), amine (-NH_2) or carboxyl (–COOH) groups. Note that there are several other functional groups that can be generated on the surface such as carbonyl, thiol, aldehyde, phosphate and silane (Goddard & Hotchkiss, 2007). In any case, surface modification must be carried out with great care. For example, if initial surface functionalization does not generate enough surface reactive groups, the compound (protein) can lose activity when placed in direct contact with hydrophobic polymer surface (Fig. 2.1A). On the other hand, too many functional groups can cause steric repulsion between proteins, which can also cause protein deactivation (Fig. 2.1B). For those reasons, the polymer functionalization must be completed with a high degree of control over surface properties upon potential chemical and physical treatments (Goddard & Hotchkiss, 2007).

Another novel strategy to design the reusable functionalized platform for protein immobilization is a reversible protein interaction on the biosensor surface. Such systems have been developed on silicon nanowire field-effect transistors (SiNW-FETs) as promising devices with their ultra-sensitivity, multiple use and label-free selective capabilities (Chen et al., 2011).

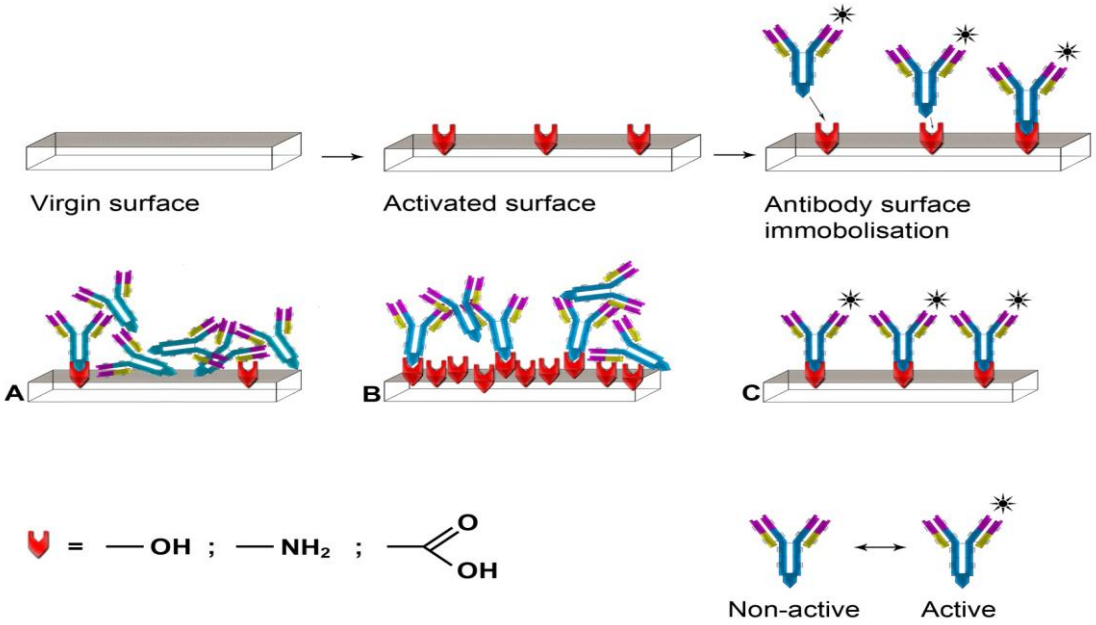


Figure 2.1: Bio-activation strategy for production of polymer detection platforms in biosensors, (A) insufficient concentration of functional groups; (B) overly functionalized surface and; (C) optimal concentration of surface functional groups.

In such cases, the existence of surface functional groups prior to protein immobilization is highly desirable. In recent work by X. Duan et al. the SiNW-FETs have been used for quantification of kinetics of protein interactions by translation of analyte-surface interaction into an electrical signal for high sensitivity measurements (Duan et al., 2012). The authors have used amine-functionalized SiNW with 2.9nm polyethylene glycol (PEG) spacer in order to effectively immobilize biotin for investigation of biotin/streptavidin interaction (Duan et al., 2012). Whether the biosensor detection is performed on polymeric surfaces (chips) or other types of devices (nanowires) the existence of the surface functional groups still presents an important feature of biosensors. In any case, one should be aware of analytical challenges in establishing the exact conformation of proteins on the surfaces used in biosensor applications (Trilling et al., 2013). The existing way to analyze the effectiveness of biosensors is only

through direct verification of protein activity once the immobilization has been performed, regardless of the type of detection and signal transduction applied in biosensors development (Coad et al., 2013; Kirsch et al., 2013).

It is clear that the polymer surface functional groups play the most important role in protein immobilization. Polymeric materials offer such a possibility as they can be processed and designed by means of synthetic procedures and with controlled surface chemistry. The most common polymeric materials for biosensor devices are polystyrene (PS), polydimethylsiloxane (PDMS), polyethylene terephthalate (PET), polycarbonate (PC), cyclic olefin copolymer (COC) and polymethyl methacrylate (PMMA) (Liu et al., 2007). Table 2.1 shows the advantages and disadvantages, together with some recent applications of these substrates. The major advantage of polymeric materials is their low cost, processability on the mass scale and the possibility to integrate microelectronics and microfluidic technology onto the surface of designed chips (Ahn et al., 2004; Fixe et al., 2004b; Hu et al., 2013; Bai & Lee, 2006). Another important advantage is the high transparency, which enables particular polymer systems that utilize optical detection. In comparison to polymer systems for developing fluorescence-based diagnostic devices, PMMA has many advantageous properties such as low intrinsic fluorescence, transparency and versatility in fabrication (Sok et al., 2009). Microfluidic devices are portable, low cost microfluidic platforms designed to automate one or more analysis steps such as mixing, separation and sedimentation into one monolithic device. Such devices act as a miniaturized laboratory system that can be used in remote and/or rural areas for detection of multiple viruses without human involvement, which, in turn, makes the detection procedure of fatal diseases safer for laboratory (Baba, Vidergar, & Marcello, 2014; Lee et al., 2014; Teles & Fonseca, 2015; Vercruysse et al., 2015).

Microfluidic devices based on plastic materials show great promise due to their versatility, the small amount of sample required for analysis and the possibility of high throughput screening (Liu & Li, 2012). Miniature devices called Biological Micro-Electro-Mechanical Systems (BioMEMS) have shown important biomedical applications such as high sensitivity diagnostic devices (Bashir, 2004; Henares 2008; Lai, 2004). BioMEMS can be defined as ‘‘devices or systems, constructed using techniques inspired from micro/nano-scale fabrication, that are used for processing, delivery, manipulation analysis, or construction of biological and chemical entities’’ (Rusmini, 2007; James et al., 2008; Qu et al., 2004; Ziaie, 2004). Many BioMEMS systems are based on PMMA material and other polyacrylate systems and in most cases the surface modifications are required since the surface of PMMA is not reactive and hydrophobic (Rusmini, 2007).

Table 2.1: Development of polymeric substrates for diagnostic biosensor applications.

Polymer	Advantages	Disadvantages	Recent applications
PMMA	Low intrinsic fluorescence High optical transparency Low cost High scratch resistance Versatility in fabrication Impact resistance	Limited compatibility with organic solvents	Microfluidic biosensor with interdigitated ultra-microelectrode (IDUA) arrays for electrochemical quantification (Wongkaew, et al., 2013)
PS	High optical transparency Chemical inertness Commercially available in different colors Low cost	Limited compatibility with organic solvents Ineffective barrier to oxygen and water vapor	High refractive index coating for label-free long period gratings (LPG) biosensor (Pilla et al., 2011)
PC	Thermal molding Durability and high strength High optical transparency Commercially available	Low scratch resistance	A microfluidic electrochemical biosensor chip for micro flow-injection amperometric determination of glucose (Wang et al., 2010)
PET	Recycled material Semi-transparent Moisture barrier Semi-crystalline Impact-resistant Light weight and flexible	Discoloration over the period of degradation	The silver-nanoparticles-on-plastic (PET): a localized surface plasmon resonance (LSPR) biosensor (Fan et al., 2010)
COC	Good performance in micro fabrication High optical transparency High moisture resistance Low dielectric constant High heat resistance	Relatively expensive Limited data available in literature	On-chip DNA, LSPR detection from gold nano-ring coated substrate integrated with COC based microfluidic device (Huang et al., 2012)
PDMS	Elastomeric material Good performance in micro fabrication Non-toxic	Gas-permeable Limited compatibility with organic solvents Degree of elasticity is temperature dependent	PDMS/paper/glass hybrid microfluidic biochip for one-step multiplexed pathogen detection (Zuo et al., 2013)

The surface of the PMMA can be modified in many different ways (both chemically and physically) while maintaining both transparency and original mechanical properties. Although several approaches are available to modify the PMMA surface for protein detection, the major strategy is to chemically functionalize PMMA plastic by surface treatment and introduction of hydroxyl (–OH), carboxyl (–COOH), or amine ($–NH_2$) reactive groups (Goddard & Hotchkiss, 2007), as depicted in Fig 2.2. Those functionalities can be further reacted in order to immobilize (both physically and chemically) proteins in biochips and microfluidic devices produced from PMMA (Liu et al., 2010). In this perspective, some recent advances in the design and surface modification of polyacrylate materials (mainly PMMA) for biosensor applications.

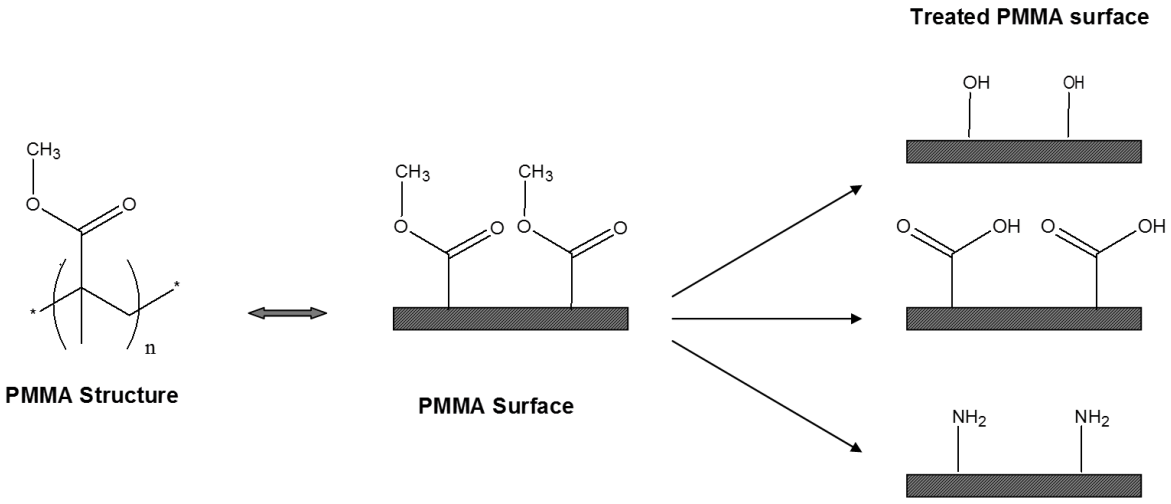


Figure 2.2: Functionalization of PMMA and expected outcome with reactive surface groups: (top) hydroxyl; (middle) carboxyl; and (bottom) amine functional groups on treated PMMA surface.

Discussion is divided in three general parts, reviewing strategies for the polymer surface treatments such as: (i) wet chemical treatment; (ii) plasma treatment; and (iii) UV radiation and photo-grafting.

2.3 Functionalization techniques

2.3.1 Wet chemical treatment and creation of polymer surface functional groups

Wet chemical treatment methods of PMMA surfaces in biosensor applications can be generally divided in four approaches: (i) acid or alkaline treatments; (ii) amine functionalization; (iii) glutaraldehyde treatment of aminated surfaces; and (iv) siloxane treatment by sol-gel technique. The expected result of all wet chemical treatments is a polymer surface with reactive functional groups for further protein immobilization (Cheng et al., 2011; Ciampi et al., 2010; Langley & Fairbrother, 2007; Rohr, 2003). Possibly the major advantage of this classical approach is that the method does not require specialized equipment. Another important advantage is that the reagent could penetrate porous three-dimensional (3D) substrates important for functionalization of microfluidic devices (Goddard & Hotchkiss, 2007; Maaz et al., 2007; Riwotzki & Haase, 1998; Wu et al., 2005).

Base and acid hydrolysis have been used to generate carboxylic acid groups on PMMA with concentrated sodium hydroxide and sulfuric acid (Brown et al., 2006; Varma et al., 2003). In particular it has been reported that 16 h treatment in 10M sodium hydroxide at 40 °C resulted in 0.66 nmol/cm^2 carboxylic acids on PMMA (Goddard & Hotchkiss, 2007). Other reports also describe the chemical conversion of PMMA methyl ester side chains by various chemical agents (Cheng et al., 2004; Fixel, 2004b; Goddard & Hotchkiss, 2007). For example, Y. Liu et al. have used base treatment in order to produce PMMA microfluidic chips coupled with electrochemical detection system for detection of trace levels of α-fetoprotein hepatocellular carcinoma biomarker (AFP) by immobilizing AFP antibody on the PMMA microfluidic channels (Liu et al., 2009). The microchips were first treated with 1M NaOH solution at 55 °C for 30 min in order to produce –OH surface groups and the chips were subsequently immersed in

poly(ethyleneimine) (PEI) solution (0.2%, pH=7.0) at room temperature for 1 h to derive $–NH_2$ for further protein attachment (surface characterization of PMMA following surface treatment has not been reported) (Liu et al., 2009).

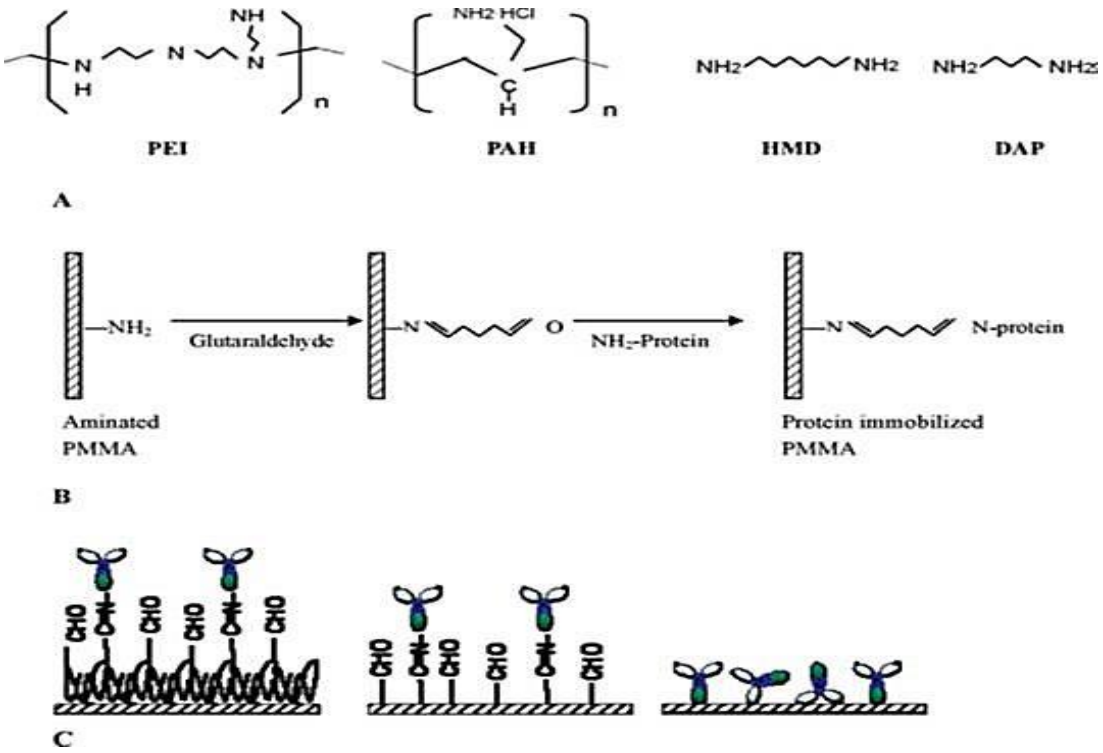


Figure 2.3: Antibody binding on different PMMA surfaces treated with different amine-bearing chemicals: (A) chemical structures of PEI, PAH, HMD, and DAP amine spacers; (B) steps in PMMA surface treatment and subsequent antibody binding: amination, cross-linking with GA and covalent binding of antibody; (C) illustration of the spatial effects of different surface modifications on antibody binding on the PMMA surface (with permission from Crowley & Pizziconi, 2005).

Amine-terminated molecules of different sizes and structures are often used as an intermediate compound between the PMMA surface and the actual protein. Those molecules are usually called “spacers” and they provide a useful way to preserve the activity of immobilized protein (Fig 2.3). The chain length and structural morphology of amine-terminated linkers is an important parameter to consider for potential protein immobilization on biochips or BioMEMS devices. In order to examine the effect of amine structure on protein activation and detection

sensitivity of microfluidic immunoassay chips, Y. Bai et al. have developed a microfluidic immunoassay ELISA device produced from PMMA (140 µm wide, 125 µm deep, and 1.5 cm long) by using a computer numerically controlled (CNC) machine (Fig 2.3). A surface wet treatment method was introduced using different amine-bearing polymers: PEI, poly(allylamine hydrochloride) (PAH), hexamethylenediamine (HMD), and 1,3-diaminopropane (DAP), in order to enhance antibody binding on the PMMA surface (Fig 2.3) (Bai & Lee, 2006). For PEI or PAH treatment, PMMA plates were first treated in 1 N NaOH and then immersed in a PEI or PAH solution (0.2%, pH 7) at room temperature for 1 h. For HMD and DAP treatment, PMMA were immersed directly in polymer solutions without prior NaOH treatment. Aminated PMMA plates were subsequently treated with glutaraldehyde for antibody binding as depicted in Fig 2.3. By treating the PMMA surface of the micro-channel on the microfluidic device with longer amine spacer such as PEI, antibodies can be bound to the micro-channel surface with antibody activity preserved. As compared to the surfaces treated with the small amine spacer molecules (HMD, DAP, Fig. 2.3), both PEI and PAH were able to significantly increase the distance between the antibody and the surface thus afforded the antibodies sufficient spatial freedom. The larger spacer also allowed more antibodies to bind on the surface thus increasing the sensibility of potential diagnostic device: PEI-treated PMMA micro-channels gave higher fluorescence signals with improved signal/noise ratio and performed better for ELISA applications (Bai & Lee, 2006). However, use of spacers, in general, may present more complicated approach than direct binding of proteins thus requiring specialized expertise (Coad et al., 2013).

The precise surface patterning of proteins is critical in the development of micro analytical biosensor systems. The major goal is to create a selective biochip

device that enables high-affinity binding to analytes of interest (Lion et al., 2003). Sol-gel technology, a technique considered to be a relatively mild route for the immobilization of proteins, has been used to generate protein microstructures on the solid polymer or glass supports (Gill & Ballesteros, 2000). In this particular process the biomolecules are entrapped in the swelling gel network instead of being chemically attached to the surface functional groups (Fig 2.4) (Gill & Ballesteros, 2000). In terms of protein stability, it has been reported that sol-gel entrapment provides improved resistance of biomolecules towards chemical and thermal denaturation (Soper et al., 2002). For those reason there is a promising future for utilization of sol-gel technique in production of robust biosensor devices. However, there are two important factors that surface scientist must consider in terms of sol-gel protein immobilization onto solid PMMA surface: (1) in case of relatively hydrophobic plastic surfaces (such as PMMA), direct contact with proteins might cause protein denaturation; and (2) because there is no intermolecular interaction between gel matrix and PMMA substrate, it is necessary for the development of routine, simple, and well-defined surface functionalization of PMMA and modification protocols in order to improve the biomolecule immobilization entrapped within gel system (Keller et al., 2012; Qu et al., 2004; Soper et al., 2002). For those reasons, gel systems with entrapped active proteins must be appropriately anchored to the solid surface through created surface functional groups such as silane functionalities for silicone gel anchoring. H. Qu et al. have designed a graft copolymer system in order to introduce the silane functional groups onto the plastic PMMA surface (Fig 2.4) (Qu et al., 2004).

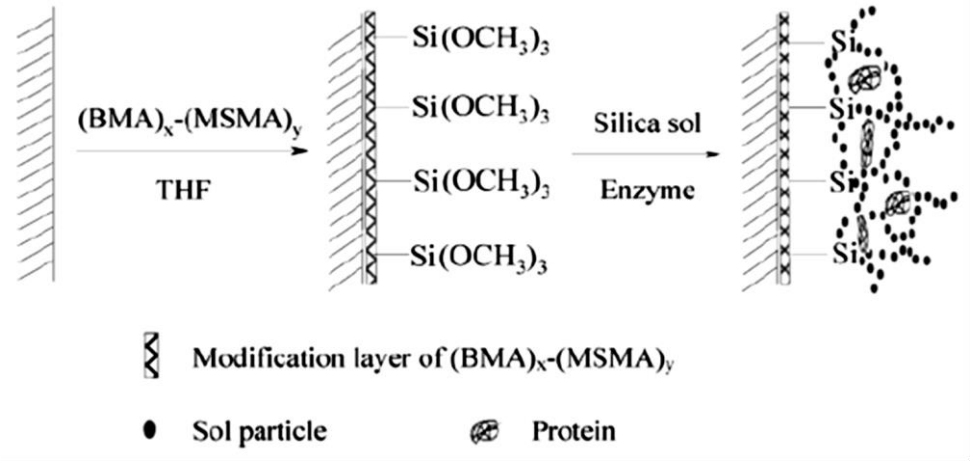


Figure 2.4: Process summary of the functionalized PMMA with silane groups originating from the $(BMA)_x$-$(MSMA)_y$ copolymer coating as a surface modification layer. The copolymer coating of PMMA substrate is followed by sol-gel protein immobilization (with permission from Jung et al., 2008).

For that purpose authors have reacted butyl methacrylate (BMA) and methylacryloxy-propyl-trimethoxysilicane (MSMA) in well-defined free radical polymerization reaction by using azobisisobutyronitrile (AIBN) as a free radical initiator. The concentration of pending silane functional groups from the resulting $(BMA)_x$-$(MSMA)_y$ copolymer was controlled by simple variation of the initial BMA/MSMA molar ratio (x/y) in free radical polymerization. The control over the copolymer composition provides an optimal concentration of pendant silane groups (Fig 2.4). The schematic view of $(BMA)_x$-$(MSMA)_y$ modification of PMMA surface and subsequent immobilization of encapsulated proteins within silica gel is presented in Fig 2.4. With this technique a physical immobilization of proteins can be achieved onto the hydrophobic PMMA micro-channels with bioactivity preserved as far as possible. Due to a relatively large micro-structured surface area hydrophilicity, and cross-linked silica gel morphology, the encapsulation of protein in this matrix has a strong potential for high biomolecule

loading and an increase of detection sensitivity through preserved bioactivity remaining in the microfluidic device (Qu et al., 2004).

Although wet chemical treatment might appear as a cost-effective and simple method, there are considerable disadvantages of those methods. One of the major concerns are that the wet chemical methods could often be non-specific, generating undesirable irregular surface etching (Goddard & Hotchkiss, 2007). Furthermore, acid or alkaline treatments can produce a range of oxygen-containing functional groups on the plastic surface. In particular the chemical (wet-treated) PMMA ester conversion of side chains will strongly depend on the side chain orientation. Another important aspect is the stability of chemically modified surfaces. For example, siloxane linkages (described in previous paragraph) can be hydrolyzed in alkaline solutions or in elevated temperatures (Wasserman et al., 1989). When more specific methods of detection such as sandwich ELISA is aimed, it is also possible that entrapped primary antibodies could not be eliminated in washing procedure hence produces a wrong signal as a results of coupling with labeled secondary antibodies. It is important to note that in sandwich ELISA the analyte of interest is sandwiched between two antibodies from both sides. Such protocol minimizes the chance of non-specific bindings in the assay. There is an environmental concern about generation of toxic waste when wet chemical treatments are applied in industrial scale. For the above mentioned reasons, the efforts in scientific research have also been and still are focused on alternative methods for surface modifications through plasma and radiation treatments.

2.3.2 Plasma surface treatment of PMMA platforms for protein immobilization

Possibly one of the major advantages of plasma treatments of plastics such as PMMA over wet chemical methods is that the plasma treatment can provide modification of the top nanometer layer with much less roughening and surface degradation (Chan, Ko, & Hiraoka, 1996). Another important advantage is that plasma treatment does not involve toxic and corrosive solvents and therefore can be utilized on industrial scale without major concerns about the environmental impact (Hegemann et al., 2003; Ihara 2002; Lai et al., 2006; Liu 2002; Morent et al., 2008; Yang et al., 2002). There are several types of plasma functionalization of plastic surfaces and the imparted functionalities can be varied by selection of precursor gases in plasma treatments and the operating parameters (precursor flow rate, pressure inside the plasma chamber, power and time of the sample exposure) (Djordjevic et al., 2008; Lane & Hourston, 1993). Many different gases have been used in plasma treatments such as: Ar, O_2, N_2, CO_2, NH_3, as well as vapors from H_2O and various liquid amines (Bhattacharya et al., 2005; Cai et al., 2008; Höcker, 2002; Kitova, 2005; Sladek et al., 2004; Ting et al., 2010). The major idea remains the same as for the wet chemical surface treatments generation of reactive functional groups such as amine ($–NH_2$), carboxyl (–COOH) and hydroxyl (–OH) for further protein immobilization is essential. Functional groups such as aldehyde or epoxide can also be achieved through "plasma polymerization" for subsequent coupling of proteins with "gentle" methods that would retain protein function, in some particular cases (Coad et al., 2013).

Plasma polymerization methods offer many advantages such as: single-step dry surface functionalization, fabrication of thin functional coatings on materials of different shapes and sizes, and uniformity of the coated layers (Mishra et al.,

2011). There are many biological applications of plasma polymerization including fabrication of both bio-reactive and non-bio-reactive coatings for microfluidics, cell culture substrates, regenerative medicine and tissue engineering (Hernon et al., 2006; Mishra et al., 2011). Another important aspect is that plasma treatment changes the surface from hydrophobic to the hydrophilic state; a very important feature for the protein stability on the plastic surface (Goddard & Hotchkiss, 2007).

Generally, PMMA surface is considered to be hydrophobic in terms of protein-surface interaction. The reported water-in-air contact angle values for PMMA plastic are in the range of 80°-90°. For example, in antibody/antigen immobilization techniques for diagnostic microfluidic devices, the interaction of antibodies with hydrophobic surfaces may cause protein denaturation leading to poor protein activity (Liu & Li, 2012). Making a surface protein-compatible is the necessary step in any type of surface modification including the plasma treatment. Plasma treatment creates the desired charged groups and increases the "overall surface energy", which in turn also increases hydrophilicity and wettability (Rusmini, 2007). Through the generation of polar functional groups on the polymer surface, hydrogen or covalent bonds may be formed across the interface thus strengthening the bonds between the two contacting surfaces (Coad et al., 2013). Y. N. Tennico et al. reported an improved bonding method via surface modification of plastic and glass materials. PMMA surface was treated in oxygen plasma in order to produce –COOH groups on the polymer surface as illustrated in Fig 2.5 (Tennico et al., 2010). Immediately following the plasma treatment, the surface was silanized by wet chemical treatment (10% tetraethyl or thiosilicate TEOS solution in 60:40 (v/v) isopropyl alcohol/water mixtures). The silane-functionalized surface was activated a second time with the oxygen plasma

under the same experimental conditions as the first plasma treatment. Although the authors used this technique for physical bonding (gluing) of polymer surfaces for fabrication of microfluidic channels, it is important to note that the reported functionalization method could also be used for protein immobilization through either covalent binding (via –COOH groups) or physical encapsulation via sol-gel technique (Fig 2.5). One of the major concerns in plasma treatments of polymers (such as PMMA) is the surface ageing. Plasma-generated functional groups are generally not stable with time as the high-energy plasma-treated polymer surface tends to "relax" to its untreated state.

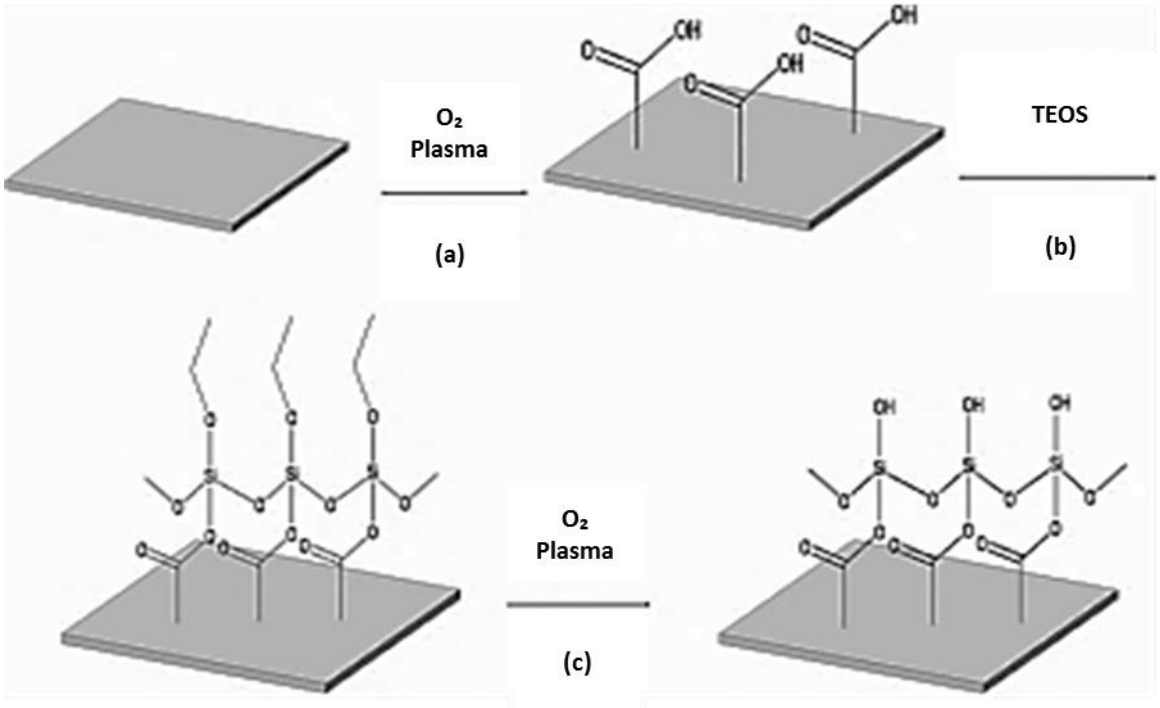


Figure 2.5: Presentation of the PMMA surface modification using oxygen plasma and TEOS. Secondary oxygen plasma treatment has been performed in order to activate the PMMA surface for physical bonding (gluing) of polymer surfaces and fabrication of micro-channels in microfluidic device (with permission from Tennico et al., 2010).

Reports indicate that oxygen-treated PMMA results in more hydrophilic –COOH groups so the aging can affect induced generated functionalities and thus compromising their performance in terms of the protein activity immobilized on

the treated PMMA surface (Goddard & Hotchkiss, 2007; Lane & Hourston, 1993). Generally, the ageing effect of plasma-treated polymers is dependent on different storage conditions such as humidity, temperature etc. The major processes responsible for surface ageing are: (i) the re-orientation of the polar groups on the surface; and (ii) the mobility of small polymer fragments into the polymer bulk. In either case, the result of aging is usually the loss of surface activity and decrease in surface energy. A. Vesel and M. Mozetic have observed the ageing effect on PMMA surface after oxygen plasma treatment. The ageing experiment was performed in order to compare different plasma treatment times and storage conditions: surface was characterized by contact angle experiment, atomic force microscopy (AFM) and x-ray photoelectron spectroscopy (XPS) for the PMMA samples stored in water and air (Vesel & Mozetic, 2012). The oxygen-plasma treated PMMA samples were also stored in different temperatures in order to establish the temperature effect on ageing. The authors reported that increased treatment time induces higher crystallinity at the surface thus decreasing the rate of aging significantly.

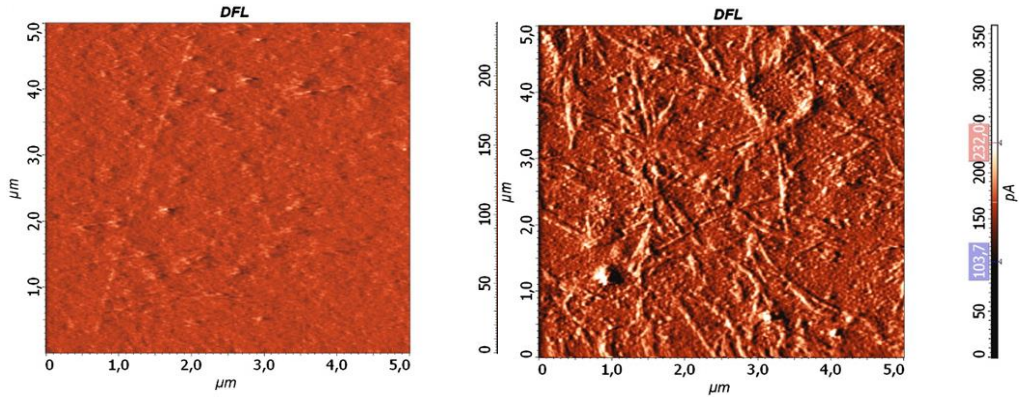


Figure 2.6: AFM investigation of the protein immobilization effect on the surface morphology of PMMA. Left: virgin PMMA surface. Right: PMMA with immobilized streptavidin through RFGD plasma-generated functional groups on treated PMMA surface (with permission from Vesel et al.,2012).

The high crystallinity induces limited surface mobility and therefore prevents the surface re-organization after the treatment. As expected, the highest aging effect was observed for the samples stored in water and on elevated temperature (Vesel et al., 2012; Vesel & Mozetic, 2012). Note that the further protein immobilization onto plasma-treated PMMA surface also causes alteration in the surface morphology (measured with AFM; Fig. 2.6) (Vesel et al., 2012). As it was mentioned at the beginning of this section, different precursors in plasma treatments can be used for PMMA surface functionalization. For example, ammonia and nitrogen plasmas have been used to impart amine groups to the polymer surface, while inert gases can be used to introduce radical sites on the polymer surfaces thus introducing the reactive sites for subsequent graft polymerization (Chevallier et al., 2005). For instance, polymers can be pre-treated with Ar in order to activate the surface for further graft polymerization of acrylic acid (AA) thus producing a polymer coating with surface –COOH groups (Kang et al., 1996). E. T. Kang et al. pretreated PTFE (Teflon^{TM}) with Ar plasma in order to graft polymerize AA on PTFE surface. In a process called radio frequency glow discharge (RFGD), plasma is used to produce reactive species from vaporized monomers and thus induce a subsequent graft polymerization (Biederman et al., 2001). One of the most important advantages of RFGD treatments is that it can be used to modify surfaces of porous structures without compromising the bulk properties of the material (Djordjevic et al., 2008). Apart from RFGD there are other technical approaches in ionized-gas/plasma treatments such as Low Pressure chemical vapor deposition processes (LPCVD) process where the materials (precursors) are deposited at low pressure conditions. The result is usually a more uniform layer of material; however, a high temperature is required so this fabrication process is not suitable for some

thermally unstable precursors. Contrary to LPCVD, the plasma enhanced chemical vapor deposition (PECVD) allows reactions in plasma chamber at lower temperatures. In this technique the gases/vapors (precursors) are ionized by plasma within the evacuated chamber and subsequently deposited onto polymer substrate. The major drawback of PECVD is that the films (coatings) produced by this processes could result in questionable quality (James et al., 2008).

One of the considerable disadvantages of plasma treatments are reorganization of the surface and ageing effect. Apart from that, the vacuum technology such as plasma treatment requires a close control over many parameters involved in optimization, which might cause serious complications when intended for industrial scale. Other than that, plasma treatment results are sometimes difficult to repeat between the laboratories due to the complicated process requirements (Kitova, 2005). The most important parameters include reaction time, process temperature, chamber pressure, orientation of the reactor, distance between the substrate and electrode and gas/precursor flow rate (Djordjevic et al., 2008). There is also a substantial risk of contamination due to the high sensitivity of the method so for that reason plasma chamber should be cleaned thoroughly after each alteration of the reaction gas or the chemical precursor (Goddard & Hotchkiss, 2007).

2.3.3 UV treatment and polyacrylate photo-grafting

One of the accepted approaches in polymer surface activation is an exposure to UV light. The expected outcome of such treatment is generation of intermediate reactive sites. These semi-stable active sites can become functional groups in second stage of the treatment depending on the chosen gas, which has been applied for surface treatment (Gassan & Gutowski, 2000; Goddard & Hotchkiss,

2007; Khan et al., 2006; Li, Kim, & Sham, 2005; Xin, Daoud, & Kong, 2004). Generally, the exposure of PMMA to UV light should result in formation of the surface –COOH groups that are later utilized for protein immobilization via carbodiimide chemistry. Nugen et al. have developed the disposable electrochemical microfluidic biosensor based on PMMA plastic. The strategy was to immobilize cystamine protein onto thiolated PMMA surface previously treated with UV light (Fig 2.7) (Nugen et al., 2009). The surface concentration of –COOH groups have been measured with toluidine blue assay. A contact angle measurement has also been performed to analyze the wettability of the samples (Li et al., 2005a; Li et al., 2005b Nugen et al., 2009). The results have been compared for both UV and UV/ozone treatments and it has been found that the UV treatment (without ozone) causes much higher decrease in contact angle in comparison to the virgin PMMA, which is directly related to generation of hydrophilic –COOH groups on the PMMA surface (Nugen et al., 2009).

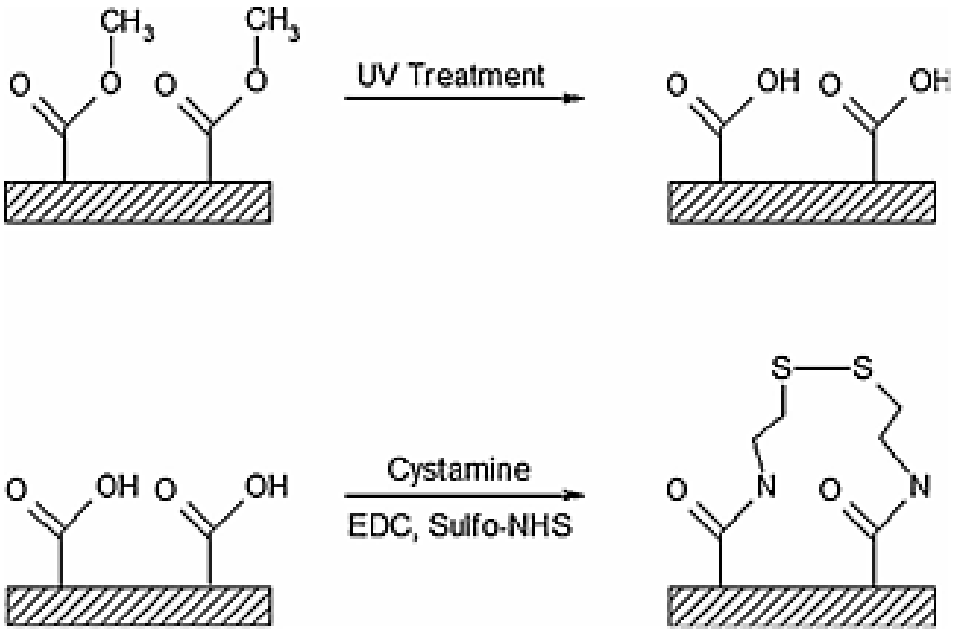


Figure 2.7: Surface functionalization PMMA with UV irradiation and the subsequent carboxylic acids conjugation to cystamine using carbodiimide (EDC/sulfo-NHS) immobilization protocol (Brown et al., 2006).

In regards to the UV treatment of PMMA surfaces, the surface concentration of generated –COOH groups is strongly dependent on the exposure time. C. Situma et al. have performed surface modification of PMMA by UV treatment for subsequent fabrication of DNA microarrays. The treatment time ranged from 5 to 30 min. The surface concentration of –COOH groups was measured and the results show saturation within 15 min. Another important finding was that the surface density of immobilized protein was found to be decreased after 20 min of UV photo-oxidation. Such behavior was attributed to the PMMA surface degradation at long exposure times (Situma et al., 2005). According to the report, the main-chain scissions of methyl ester groups are, most likely, the result of high UV doses (Situma et al., 2005). For those reasons, high exposure times are not desirable due to the high degradation rate, which reduces the number of methyl ester groups converted to –COOH groups by UV photo-oxidation (Situma et al., 2005). Another type of introducing –COOH functionalities on the polymer surface is UV-induced photo-grafting (Rohr, 2003). This reaction is important for the close control over the surface concentration of immobilized proteins and creation of protein gradients on the polymer surface. B. Li et al. described the method of graft polymerization AA on pre-irradiated PS surface (Li et al., 2005a; Li et al., 2005b). In their work, peroxides were generated on the substrate (PS) surface by UV pre-irradiation and they initiated graft polymerization of AA onto the surface upon a second UV irradiation (Fig. 2.8). A PET mask was used to create surface gradient of polyacrylic acid (PAA) as PET film can be used to cut-off UV light with a wavelength below 310 nm. The proteins were immobilized to PAA-grafted surfaces by the coupling reaction between $–NH_2$ groups of proteins and –COOH groups on grafted chains by carbodiimide chemistry (Fig. 2.8) (Li et al., 2005a, Li et al., 2005b).

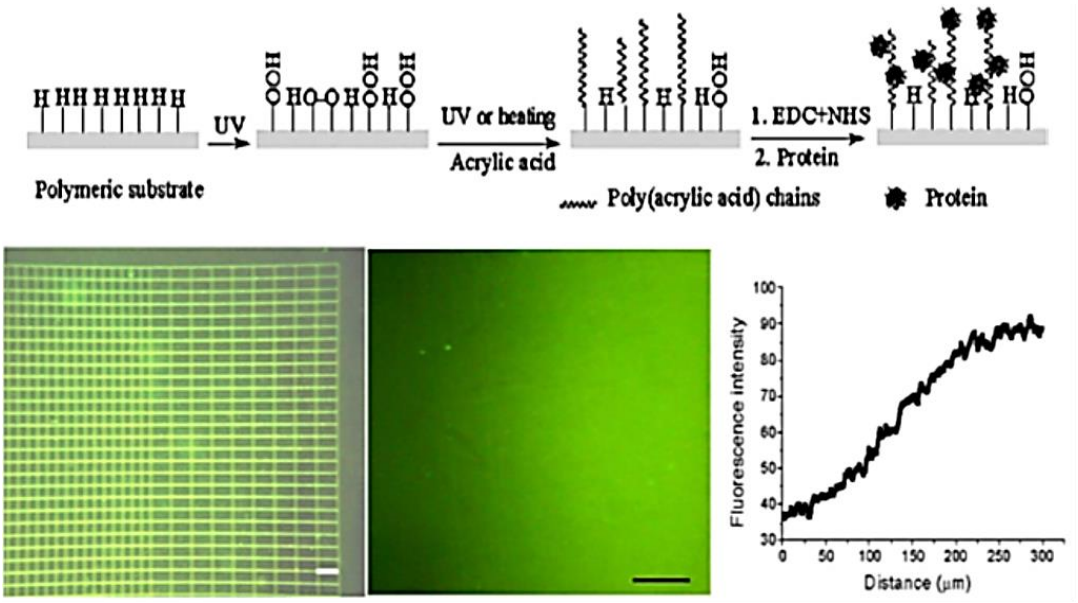


Figure 2.8: Top: illustration of the two-step UV irradiation method for grafting AA onto polymeric substrate surfaces followed by covalent carbodiimide (EDC/NHS) immobilization of protein to –COOH groups present on UV-grafted PAA molecular chains. Bottom (left): fluorescence microscopic image of micro-patterned PAA conjugated with fluorescent-labeled avidin. Bottom (middle): fluorescence microscope image showing the density gradient of avidin on PET surface (scale bars, 50 μm) (with permission from Cheng et al., 2004).

Although, in this work authors have created protein gradients in order to observe fundamental cell properties when attached on bio-activated polymer surface, the reported method can be used for creation of biosensor devices with the high level of control and potential increase of detection sensitivity (Li et al., 2005a; Li et al., 2005b). One of the major advantages of photo-grafting process is that the surface modification can be carried out under mild conditions thus eliminating the need for high energy consumption, expensive equipment and excessive use of solvents (Akbari et al., 2003; Pu et al., 2007; Qin et al., 2009; Rohr, Hilder et al., 2003; Roy et al., 2011; Wu et al., 2006; Yang & Yang, 2003). The surface activation with proteins can be performed in only few steps such as: UV light irradiation, carbodiimide coupling and washing (Li et al., 2005a; Li et al., 2005b). According to B. Li et al. the photo-activation method makes array fabrication simple and less time consuming and does not require extensive amounts of specialized

reagents to produce robust linkages with high surface densities (Li et al., 2005a; Li et al., 2005b). Another important characteristic is that the active functional group is introduced with simultaneous polymerization. For that reason no additional activation process is required for covalent immobilization such as alkaline/acid treatment or plasma activation. Furthermore, there is an indication of lower detection limits reported for photo-grafted epoxy resin-based immunoassay devices (Situma et al., 2005). However, UV treatments can affect the optical properties of the treated polymer. There is also a question of polymer degradation at the surface and creation of unstable functionalities that would affect the storage capacity due to the surface relaxation and ageing effect.

2.4 Immobilization techniques

Variety of techniques have been reported for immobilization of enzymes, antibodies, antigens and different types of proteins on the supporting surfaces (Goddard & Hotchkiss, 2007; Jonkheijm et al., 2008; Liu & Li, 2012). Immobilization techniques have been commonly applied for food industry, organic compound removal from wastewater, in situ measurements of environmental pollutants, metabolite controls and biosensor applications (Khan & Alzohairy, 2010). Table 2.2 highlights the examples of well-known immobilization techniques and applications of such techniques for biomolecule immobilization.

Maybe the most straight forward method for immobilization of biomolecules for subsequent biorecognition is physical immobilization. This method relies on the physical attachment of the protein to the surface, which is commonly used in clinical practices such as ELISA. In this technique immobilization mainly occurs through different interactions between targeted biomolecules and solid substrates (Rao, Anderson, & Bachas, 1998; YoungáJeong & HyunáChung, 2008). There are some key forces that can greatly influence the efficiency of the immobilization among which,

three important forces play the vital roles in successful immobilization. Namely ionic interaction (electrostatic interaction), hydrophobic interaction and hydrogen bonding have the great impact on the immobilization efficiency (Yoon et al., 1996). Surface functional groups of the supporting substrates (such as –COOH) can interact with functional groups of the proteins (such as $–NH_2$) and result in protein attachment through ionic attraction (Alcon et al., 2002; Xu et al., 2006).

Table 2.2: Biomolecular surface immobilization techniques and the biosensor applications.

Immobilization techniques	Applications
Physical attachment	Detection of DV by using ELISA assay (Alcon et al., 2002) and early detection of DV using ELISA analytical kit (Beckett et al., 2005)
Immobilization by entrapment	Immobilization of glucose oxidase in polypyrrole for its application in amperometric glucose sensors (Foulds & Lowe, 1986) and sol-gel-entrapped glucose-oxidase immobilization for sensing application (Avnir et al., 1994)
Covalent immobilization via carbodiimide chemistry	Modification of polystyrene particles with amine substitute HSV reporter probes via carbodiimide chemistry for ultimate DNA detection (Thomson et al., 2012)
Covalent immobilization via zero-length cross-linker	Attachment of trypsin by using glutaraldehyde as cross linker on ammonia plasma treated polyethylene films (Ghasemi et al., 2011)
Covalent immobilization via spacers	Using amine spacers of different sizes such as PEI, HMD, PAH and DAP for enhancing antibody binding to polymer based microfluidic devices (Bai et al., 2006)
Oriented immobilization	CNBr activation of sepharose (Seph) coupling to protein via the δ-amino group of lysine to form isoureas (Wilchek & Miron, 2003)

According to previous findings, the effect of ionic interaction on protein immobilization in comparison to other two forces can be considered insignificant (Yoon et al., 1996). Hydrophobic nature of the supporting substrate can offer protein immobilization via hydrophobic interaction while presence of surface –COOH groups (in the current case) can result in hydrogen bonding with the primary amines of the proteins (Yoon et al., 1996). Between these two major forces, hydrogen bonding has proven to influence protein immobilization in a stronger manner than hydrophobic interaction. Despite development of more complex immobilization techniques, clinical practice still relies on the direct attachment of the targeted biomolecules to the surface. This method is simple and cost effective as it avoids extra steps and expensive chemicals used for treatments. Relative hydrophobicity of the bioreceptor surface goes hand in hand with the existence of desirable surface functional groups that make this method more effective.

The entrapment of the antibody/antigen inside the membrane or gel material is another relatively simple method for immobilization. In this method the permeability of the material has drawn a great deal of importance as it can directly affect the sensitivity and background noise of the resulting detection signal. The major drawback of such techniques is that the unreacted entrapped primary antibody might not be eliminated during the washing step due to the structure of the supporting material, hence causing a false signal as a consequence of coupling with secondary antibody (Scouten et al., 1995; Reetz, Zonta, & Simpelkamp, 1996). Therefore this method might result in unacceptable level of background signal originated from the substrate, in particular when more complex protocols such as sandwich ELISA are aimed.

Covalent immobilization of antibody/antigen is one of the most commonly applied methods even in the routine laboratory diagnostics. In this method the existing stable functionalities are generally transformed to the semi-stable highly reactive functional groups that can covalently bind with the proteins. Covalent immobilization can be

performed via cross-linkers of different categories. A great number of research works have reported the application of zero-length cross linkers such as glutaraldehyde (GA), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS) in covalent immobilization of the proteins to the substrate (Wang et al., 2011; Sam et al., 2009). Functional conversion can occur quite differently from one cross-linking agent to another, thus making reproducibility difficult. For example, one of the most commonly used carbodiimide agents is the water-soluble EDC for aqueous crosslinking. Carbodiimides such as EDC, can activate –COOH functional groups for direct conjugation to primary amines of the biomolecule. The available –COOH surface groups can be converted to unstable reactive O-acylisourea ester groups (Figure 2.9, step 3). This intermediate compound, by association of NHS can be converted to semi-stable NHS-ester groups (Figure 2.9, step 5), which is a highly reactive intermediate compound toward $–NH_2$ groups of peptide sequences (Lys) from proteins, thus resulting in covalent immobilization of the protein on the surface.

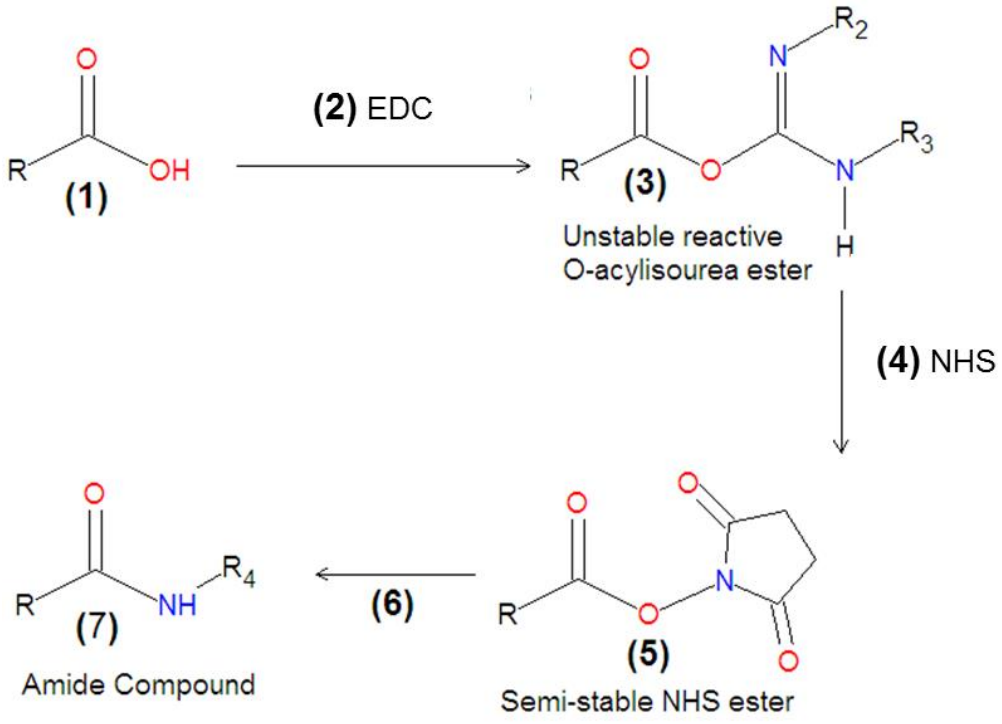


Figure 2.9: Carbodiimide reaction and activation of the –COOH functional groups.

However the versatility of resulted functional groups in EDC/NHS chemistry makes repeatability of this technique difficult. Previous reports also indicate the precautions and possible drawbacks of such approach as in some cases; EDC/NHS reaction can cause the formation of the anhydride functional groups (instead of NHS-ester groups), which are unreactive towards proteins (Sam et al., 2009). Early cross-linking inside the individual protein molecules might also happen when EDC/NHS treatment is aimed for activation of the surface. Such undesirable effects can cause the loss of protein activity that, in turn, can also result in poor detection signal and significant loss of sensitivity (Coad et al., 2013)

Larger cross-linkers are called "spacers molecules". Spacers are molecules with available functional groups for coupling with proteins. In particular, they can cause distance between proteins and the solid surface of the substrate, resulting in higher spatial freedom for binding of proteins to the surface. Among all kind of spacers, amine bearing molecules such as PEI, HMDA and DAP (1,2-diaminopropane) are the most commonly used intermediate compounds (Bai & Lee, 2006). Linear amine spacers such as HMDA are smaller in size and therefore offer less active functional groups in comparison to branched spacers such as PEI. For that reason, larger spacers normally result in higher immobilization rate due to the number of available active functionalities, which are offered for protein attachment. As the first step, carbodiimide chemistry provides the reactive intermediate functionality suitable for covalent attachment of the NHS ester groups to terminal $–NH_2$ functionalities of spacers. In a further step, the aminated surface reacts with glutaraldehyde, yielding aldehyde groups that could form imine linkages with primary amine groups of proteins (Coad et al., 2013; Wang & Jin, 2004). Glutaraldehyde (GA) is a well-known amine-reactive homobifunctional cross-linker, frequently used in biochemistry applications. Such experimental strategy results in

reactive functional groups for more robust protein binding with high reproducibility (Wang & Jin, 2004; Bai & Lee, 2006).

All of the mentioned immobilization techniques, to this point, can be classified in the category of random immobilization as there is no control over the direction of the immobilized protein on the substrate. The lack of direct control, in some particular cases, causes random immobilization that result in loss of biological activity of the antibody/antigen upon immobilization. Mainly, such loss can be attributed to the random placement of the symmetrical macromolecules on the substrate (Lu, Smyth, & O'Kennedy, 1996). Oriented attachment of antibodies to the supporting surface and subsequent increase in binding affinity can be achieved by chemical alteration of the surface functionalities (Lu, Smyth, & O'Kennedy, 1996). Therefore proteins can be attached to the surface in a very orderly manner due to this well-defined immobilization technique. This ordered way of immobilization has applications in many fields such as bioreactors, biosensors and bioelectronics. The major advantage of this method over random methods is reproducibility of the immobilization as biomolecules are immobilized through a specific site.

2.5 Directions of the current research work

Surface chemistry of the polymer substrate and protein immobilization techniques are the key issues in application of biochips and biosensors. In ideal case, the protein should retain the biological activity and provide selective interaction with other proteins of interest such as antigens/antibodies present in the blood of affected patients. In that perspective, the development of an efficient method for preparation of a polymer substrate with well-designed surface is essential. Surfaces that contain controlled concentration of functional groups could provide the preservation of the native conformation of attached proteins, which is the most important requirement for development of biosensors.

Furthermore, the optimal surface chemistry and reactivity towards protein macromolecules should lead towards highly sensitive biosensor devices, which are of a crucial importance in early diagnosis. Therefore, synthetic method for preparation of the functionalized surface has been chosen as the major aspect of this project.

The main hypotheses adopted in this project are:

- Free radical polymerization between MMA and MAA would yield copolymers with pendant –COOH groups.
- Variation of MMA/MAA can result in copolymer surfaces with controlled surface concentration of –COOH groups.
- Developed polymeric platforms with available surface –COOH groups can be successfully used for immobilization of the Dengue antibody via physical and covalent attachment and subsequent DV detection.

Considering the mentioned hypotheses, it is expected to achieve outcomes as follows:

- Determination of the chemical composition of PMMA-co-MAA and method development for production of coatings with controlled concentration of –COOH groups.
- Integration of PMMA-co-MAA coated chips into ELISA system for Dengue antibody immobilization.
- Enhanced detection signal for Dengue virus from developed biochips and improved ELISA method with highly sensitive and selective detection platforms.

CHAPTER 3

Polymethyl methacrylate-co-methacrylic acid coatings with controllable concentration of surface carboxyl groups: a novel approach in fabrication of polymeric platforms for potential bio-diagnostic devices

3.1 Introduction

The generally accepted strategy in development of biodiagnostic devices is to immobilize proteins on polymeric surfaces as a part of detection process for diseases and viruses through antibody/antigen coupling. In that perspective, polymer surface properties such as concentration of functional groups must be closely controlled in order to preserve the protein activity. In order to improve the surface characteristics of transparent polymethacrylate plastics that are used for diagnostic devices, we have developed an effective fabrication procedure of polymethylmetacrylate-co-metacrylic acid (PMMA-co-MAA) coatings with controlled number of surface carboxyl groups. The polymers were processed effectively with the spin-coating technique and the detailed control over surface properties are demonstrated through the variation of a single synthesis reaction parameter. The chemical structure of synthesized and processed copolymers has been investigated with nuclear magnetic resonance spectroscopy (NMR) and matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-ToF-MS). The surface morphology of polymer coatings have been analyzed with atomic force microscopy (AFM) and scanning electron microscopy (SEM). We demonstrate that the surface morphology and the concentration of surface –COOH groups (determined with UV-Vis surface titration) on the processed PMMA-co-MAA coatings can be precisely controlled by variation of initial molar ratio of reactants in the free radical polymerization reaction. The wettability of developed polymer surfaces also varies with macromolecular structure.

3.2 Literature review

The development of biosensors has drawn a vital role of research interest due to the high sensitivity and selectivity in detection of diseases and viruses. Of particular interests are polymeric materials used for surface protein immobilization. Those immobilized surface proteins are further engaged in detection of coupling proteins (antibody/antigen) present in diseased blood. The most commonly applied diagnostic device, based on heterogeneous antibody/antigen interaction, the enzyme-linked immunosorbent assay (ELISA), still presents a "golden standard" in clinical diagnostic practice (Lin et al., 2010; Rohr et al., 2003). However, conventional ELISA has its own limitations such as: tedious and labor-intensive protocol, long incubation times between each step and inconsistency of the results (Lai et al., 2004). In order to overcome those serious limitations, there is a strong need for development of advanced polymer coatings with controllable surface properties such as surface chemistry and morphology. In that perspective, a generation of functionalities such as hydroxyl (–OH), amine ($–NH_2$) and carboxyl (–COOH) groups at the surface of polymer coatings presents the crucial step for further immobilization of proteins and subsequent effective detection of diseases and viruses. Furthermore, the surface concentration of those functional groups must be closely controlled in order to avoid protein de-activation caused by either steric repulsion (over functionalization) or protein denaturation in close proximity of the polymer surface (low surface concentration of functional groups) (Goddard & Hotchkiss, 2007).

In recent years, polymethylmethacrylate (PMMA) has shown a great potential due to the particular properties and a wide range of applications (Liu et al., 2010). PMMA is a low cost polymer with chemical inertness, low specific weight, high

impact resistance and flexibility. PMMA has been successfully used for the immobilization of enzymes, DNA, proteins and metal particles deposition for diagnostic purposes (Bai et al., 2006; Fixe et al., 2004a; Jung et al., 2008; Saralidze et al., 2007). For developing a fluorescence based biosensor devices, PMMA has shown many advantageous properties such as transparency, low intrinsic fluorescence and ease of fabrication (Sok, Clarizia et al., 2009). The major drawback of most of the polymeric surfaces (including PMMA) is the absence of above mentioned surface functionalities. In most cases the polymeric surfaces need to be treated in order to obtain the optimum concentration of the surface functional groups (Lu et al., 2013). For example, the surface of the PMMA can be treated in various ways (both chemically and physically) without changes in transparency or in mechanical properties. Those treatments include plasma processing, wet chemical surface reactions (hydrolysis and aminolysis) or UV treatment. Although the recently reported results present an important new insight into the field of surface engineering (Coad et al., 2013), there are still major concerns about existing surface treatment techniques. For example, some authors are pointing out that the main drawback of plasma treated surfaces is ageing effect (Vesel & Mozetic, 2012). Functional groups formed on the treated surface are not stable during the time and the surface tends to return to its untreated state as the functional groups re-orient themselves (Vesel & Mozetic, 2012). The simplicity and cost effectiveness of wet chemical surface treatments seems to be compromised by non-specific reactions resulting in a range of oxygen-containing surface functional groups. Another serious concern is the surface etching, which often results in a non-regular surfaces is, difficult to control (Goddard & Hotchkiss, 2007; Wensink et al., 2005b). Similar to other existing surface treatments (such as plasma or UV treatments) the important

aspect is the stability of chemically modified surfaces and surface "relaxation" to the previous, non-treated state (Wasserman et al., 1989). Obviously there is a strong need for stable and robust materials with high level of control over surface concentration of functional groups.

In the view of the importance of polymeric surfaces for development of effective diagnostic devices, here we report the synthesis and fabrication of polymethylmethacrylate-co-methacrylic acid (PMMA-co-MAA) coatings with controlled number of surface –COOH groups. We have carefully chosen the copolymer composition for a well-established free radical polymerization synthesis protocol and subsequent surface fabrication by spin-coating technique.

The design and control over the surface chemistry of PMMA-co-MAA coatings is based on the hypotheses: (i) the variation in the initial monomer concentration of methyl methacrylate (MMA) and methacrylic acid (MAA) would yield plastic material with varying MAA segments (Figure 1.1) in the polymer chain; and (ii) the –COOH groups generated from MAA would be present at the polymer surface as well as the bulk of the material. The tuning of the polymer composition has the potential to provide a simple and effective method to control surface properties of the polymer coatings, important for their future application as diagnostic devices.

3.3 Experimental procedure

3.3.1 Chemicals and reagents

Methyl methacrylate (MMA) (Appendix Table 1), methacrylic acid (MAA) (Appendix Table 2), 2, 5-dihydroxy benzoic acid (DHB), sodium iodide (NaI), toluidine blue ((7-amino-8-methyl- phenothiazin-3-ylidene)-dimethyl-ammonium, TB) and ethanol (EtOH) were purchased from Sigma Malaysia. Deuterated dimethyl sulfoxide-d_6

(DMSO-d6) was purchased from Merk, Germany. Tetrahydrofuran (THF, Thermo Fisher Scientific, US) (Appendix Table 3), has been used as solvent in polymer synthesis and processing procedures. The free radical initiator azobisisobutyronitrile (AIBN) (Appendix Table 4) was purchased from Friedemann Schmidt Chemical, Germany. Diced silicon OFET substrates (2cm x 2cm) were purchased from Ossila, UK. MMA monomer was purified by distillation prior to the free radical polymerization synthesis (Appendix Figure 1). All other materials have been used as received.

3.3.2 Synthesis

Four different compositions of the PMMA-co-MAA copolymers were prepared by free radical polymerization reaction in THF using AIBN as an initiator. The abbreviations of the copolymers have been used identifying the initial molar ratio of the monomers. In particular, PMMA-co-MAA (9:1) corresponds to 90% of MMA and 10% of MAA in reaction mixture. MMA monomer has been purified prior to polymerization reaction. Further copolymer compositions are as follows: PMMA-co-MAA (7:3) and PMMA-co-MAA (5:5). The pure PMMA (polymerization of MMA with AIBN initiator) was synthesized in the same reaction conditions and used as control in all experiments. A three-neck round-bottom flask was fitted with a condenser and sealed inlet used for reactants feed. The set up was charged with 50 ml of THF and pre-calculated amount of MMA and stirred for 5 minutes. A mixture of MAA and AIBN (0.328g) was gradually added to the solution. Reaction was allowed to proceed for 6 hours at 90 ºC. The reaction mixture was poured into 1000 ml of distilled water. White color precipitation was filtered and washed thoroughly with water by using centrifuge. Freeze-drying has been used to remove the residual water and the samples were stored in the fridge before dissolution and subsequent chemical analysis.

3.3.3 Polymer sample preparation by spin-coating technique

The polymer coatings were prepared on silicon wafers (substrate) by spin-coating procedure. Silicon wafer substrates were previously cleaned in three following steps: (1) substrates were first immersed for 5 minutes in the solution of H_2O:H_2O_2:HCl with the ratio of 6:1:1; (2) secondly, substrates were taken out and immersed for another 5 minutes in solution of NH_4OH:H_2O:H_2O_2 with the ratio of 1:5:1; (3) the final step has been completed by rinsing the substrates in the solution of H_2O:HF with the ratio of 1:1 for 5 minutes. Silicon wafer substrates were rinsed thoroughly and sonicated in purified water for 5 minutes between each step. After washing, substrates were sonicated again and dried under stream of nitrogen. After the cleaning, silicon wafers were coated using spin-coater (model WS-650MZ-23NPP) with spinning time of 55 seconds at 3000 rpm. Coated samples were placed in oven (60°C) for solvent evaporation overnight. 5% polymer solutions (in THF) were used for all copolymer compositions including the PMMA control.

3.3.4 Polymer analysis by gel permeation chromatography (GPC), nuclear magnetic resonance (NMR), and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-ToF-MS)

The molecular weights of the polymer compositions were determined using GPC. The instrument setup consists of water 600 controllers, water 717 auto-sampler and water 2414 refractive index detector. THF was used as solvent at a flow rate of 1 $mlmin^{-1}$. The calibration of the GPC column was performed using mono dispersed polystyrene (PS) standards. Sample was dissolved in THF at around 2 $mgml^{-1}$. The ^1H NMR spectra were recorded using a Bruker NMR spectrometer (350 MHz). Polymers of four different compositions were dissolved in DMSO-d6 and placed in NMR tubes (5 mm outer diameter). The chemical shifts were measured and the peak integration has been

performed relative to the solvent peak. The polymer structures were examined with MALDI-ToF-MS analysis and the samples were deposited on MALDI-ToF-MS plate using layer-by-layer method (Djordjevic et al., 2009). In brief, 10 mg of raw polymer was dissolved in 1 ml of THF. DHB matrix was prepared by dissolving 100 mg in 1 ml of EtOH. NaI has been chosen as an ionizing agent (100 mg of NaI in 1 ml of EtOH). 0.5 µl of DHB solution was first deposited and dried on the MALDI-ToF-MS plate. A subsequent layer of NaI solution (0.5 µl) was added on the top of dried matrix. After another drying step, the third layer of the polymer solution was added on the top of the crystallized DHB–NaI mixture (0.5 µl). The sample plate was placed in MALDI-ToF-MS (ABI 4800 plus) analyzer equipped with nitrogen laser emitting at 375 nm. The detector was operated in positive ion mode.

3.3.5 Surface morphology investigation by scanning electron microscopy (SEM) and atomic force microscopy (AFM)

The surface morphology and the thickness of produced polymer coatings was analyzed with SEM equipped with a field emission gun (FESEM, JEOL, JSM7600F), which was operated at an accelerating voltage of 0.5 kV. The polymer coated samples were mounted on a double-sided conductive tape and the surface of the polymer (top) was coated with gold to avoid surface charging (the thickness of the gold coated layers: ~ 1285.2 Å). The images were recorded in secondary electron mode. AFM (Bruker, Dimension 3000) images were recorded in contact mode. Mean roughness (nm), maximum height (nm) and surface area (μm^2) were measured for all polymer compositions.

3.3.6 Water-in-air contact angle measurement

The water-in-air contact angle on the surface of polymer coated silicon wafers was measured in room temperature applying sessile drop method. A Data physics Contact

Angle System OCA instrument and imaging system have been used for this experiment. The contact angle was measured within 1, 2, and 3 minutes after a drop of water (0.1 μL) was placed on the substrate. Reported values are the average of three separate drops, on the center and two corners of the surfaces. Calculated standard deviation is presented with the results (n = 6).

3.3.7 UV-Vis titration and determination of surface –COOH groups on PMMA-co-MAA coatings

Based on the polymer structure shown in Figure 1.1, the surface of PMMA-co-MAA films were investigated for the presence of carboxyl (–COOH) groups (generated from MAA polymer segments) by spectroscopic UV-Vis toluidine blue (TB) titration. This method is based on pH dependent adsorption/desorption of ionic (positively charged, blue in color) TB molecules on negatively charged surfaces (Djordjevic et al., 2010; Sano, Kato, & Ikada, 1993). In brief, the polymer coatings were allowed to interact with TB by incubation of PMMA-co-MAA coated silicon wafers in an aqueous alkaline solution (5 mM TB in 0.1 mM NaOH) for 2 h at the room temperature; the reaction between TB and surface –COOH groups is assumed to complete in 1:1 ratio. The samples were taken out after reaction (2h) and rinsed with 0.1 mM NaOH solution for removal of the non-complexed TB dye. TB desorbs in acidic medium so after reaction in alkaline TB solution, samples were soaked in 5 ml of 50% acetic acid for 20 min in order to remove the complexed TB of the polymer coatings. The absorbance of desorbed TB in acidic solution was measured in a UV-Vis (Varian, Cary 1) spectrophotometer at 635 nm. The recorded spectroscopic data was converted into surface concentration of –COOH expressed in $\mu M/mm^2$ (n = 6).

3.4 Results and discussion

The average molecular weights (M_w) of both PMMA and PMMA-co-MAA were determined by GPC and data are displayed in Table 3.1. Identified molecular weights of all synthesized polymers were in the range of 21460-34766 $gmol^{-1}$ and the polydispersity indices (PDIs) were in the range of 1.82-2.41, indicating successful synthesis of all polymer compositions.

Table 3.1: Characterization data for synthesized PMMA and PMMA-co-MAA.

			Mol % MMA	
Composition	**M_w[a] (g mol^{-1})**	**PDI[a]**	**Exp[b]**	**Theor**
PMMA	34766	2.04	107	100
PMMA-co-MAA (9:1)	32952	1.82	89	90
PMMA-co-MAA (7:3)	33229	1.94	79	70
PMMA-co-MAA (5:5)	21460	2.41	55	50

[a]Determined by GPC; [b]determined by ^{1}H NMR

The structural compositions were determined in ^{1}H NMR experiment for all synthesized polymers. Figure 3.1 represents methylene/methyl peaks detected at 0.7 and 0.9 ppm (identified by lines in Fig. 3.1). Only in homo-polymer (PMMA, Fig. 3.1a) those peaks could be clearly separated. The splitting of methylene/methyl peaks becomes obvious with an increased concentration of MAA units in copolymer chains (Fig. 3.1b-d). The copolymer compositions, calculated as the ratios of integrated peak areas of the methoxyl groups (MMA, 3.4-3.6 ppm) and the methyl/methylene protons of both MMA and MAA segments, are presented in Table 3.1. All integration values were calculated relative to the DMSO-d_6 solvent peak. The results in Table 3.1 demonstrate a good

agreement between experimental values obtained from ^{1}H NMR and the pre-determined molar ratios in reaction mixtures (Barroso et al., 2009; Halacheva, Freemont, & Saunders, 2013).

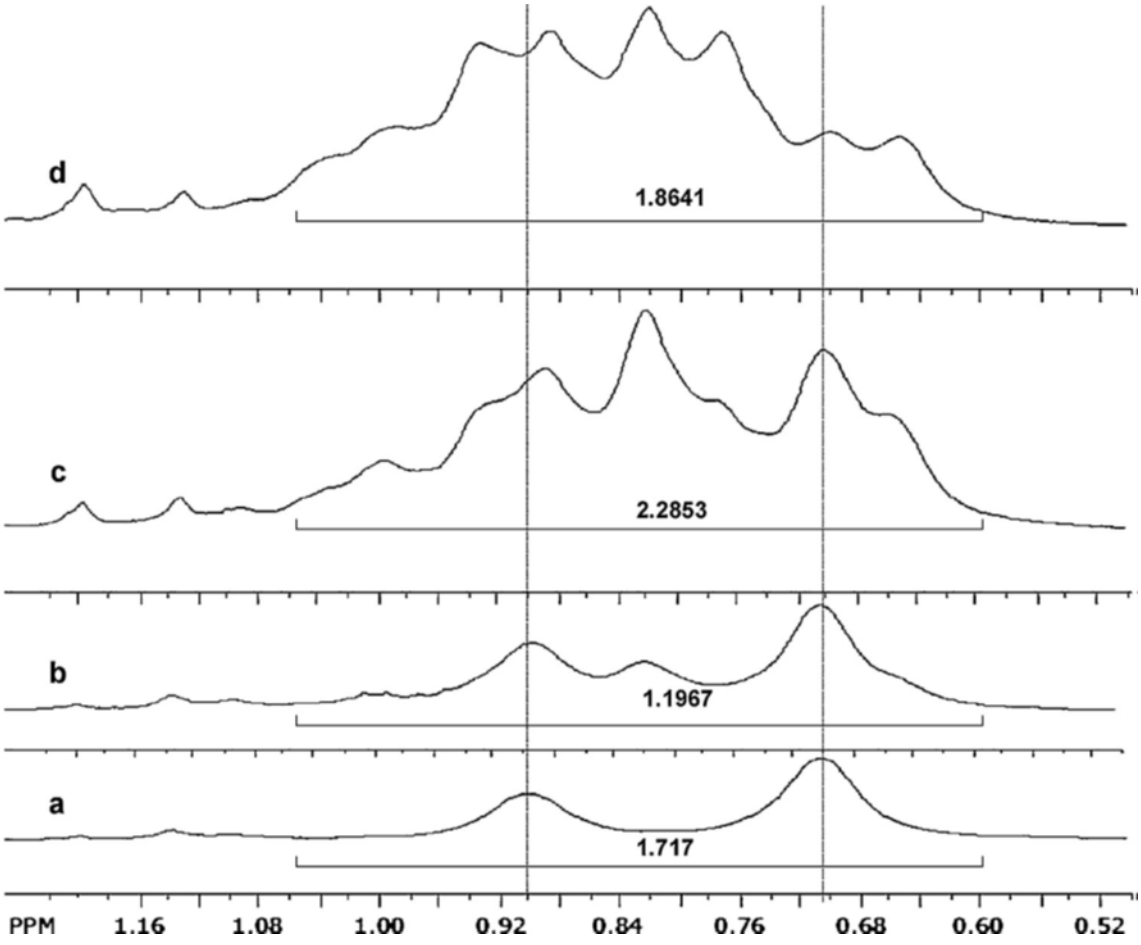


Figure 3.1: ^{1}H NMR peak detected for methyl groups of synthesized polymers; (a) pure PMMA, (b) PMMA-co-MAA (9:1), (c) PMMA-co-MAA (7:3) and (d) PMMA-co-MAA (5:5).

As a very powerful tool, MALDI-ToF-MS provides valuable information about the molecular weight distribution and the structure of macromolecules including individual oligomers and low-mass polymers (Byrd & McEwen, 2000; Jackson, Larsen, & McEwen, 1996). The MALDI-ToF-MS results are presented in Fig. 3.2 for both PMMA (control) and representative PMMA-co-MAA (5:5) copolymer. Note that the molecular weights from Fig. 3.2 do not correspond with the GPC results (Table 3.1). This disagreement is a consequence of the experimental difficulties associated with the

detection of large molecular weights with MALDI-ToF-MS (Krieg et al., 2010). The possible reason for detection of 10 times smaller molecular weights (in comparison to GPC results-Table 3.1) could be the fragmentation of large macromolecules in the ionization chamber.

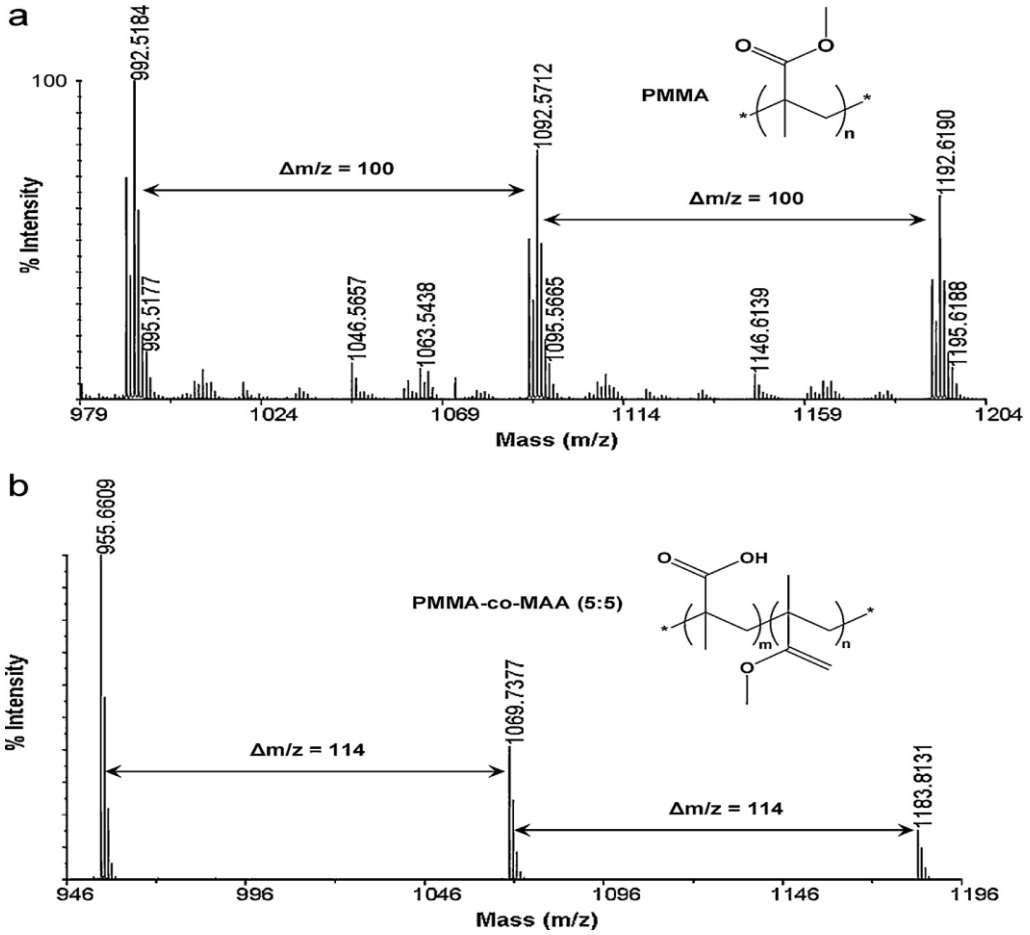


Figure 3.2: MALDI-ToF-MS spectra of synthesized polymers; (a) pure PMMA and (b) PMMA-co-MAA (5:5).

Even with the detection of relatively small polymer chains (Fig. 3.2), MALDI-ToF-MS results provide the unique picture of the macromolecular structure and the evidence of the exact distribution of monomer units, which is particularly important information for the work presented. Representative MALDI-ToF mass spectra show the molecular weights of different compositions determined for $[Pol.+Na]^+$ in the range of 955 - 1195 m/z (Fig. 3.2). For PMMA spectrum (Fig. 3.2a) the interval between peaks, Δm/z=100

corresponds to the molar mass of the MMA units (Krieg et al., 2010). For PMMA-co-MAA (Fig. 3.2b) the peak at m/z=955 was calculated as follows:

$$[(MMA)_n (MAA)_m\text{-}MAA + Na]^+ \longrightarrow 4(100) +1+5(86) +101+23= 955\ m/z$$

From the structure displayed in Fig. 3.2, b, the m/z=955 peak corresponds to n=4 and m=6 with MMA and MAA end-groups. In the second peak (m/z=1069), n=5 and m=6 with present MMA polymer end-groups:

$$[(MMA)_n (MAA)_m\text{-}MMA + Na]^+ \longrightarrow 5(100) +1+5(86) +115+23=1069\ m/z$$

The third peak (m/z=1183) corresponds to the presence of 7 units of MMA and 4 units of MAA with MMA end-groups. Figure 3.2b also represents MALDI-ToF-MS obtained for other compositions, as all obtained spectra showed similar patterns indicating succesful copolymerization with alternating MMA/MAA units. The described structural analysis clearly shows that the number of MAA units with –COOH groups can be controlled by simple variation of initial molar ratio of reactants. Particularly, the –COOH (originating from MAA) functionalities also exist on th copolymer surfaces, as explained further in this discussion.

The synthesized copolymers with defined chemical structures have been used to produce spin-coated surfaces. After careful optimization of the process parameters such as spin speed, coating time, concentration of the polymer solution, solvent evaporation kinetics and the nature of the substrate (Cui, Li, & Han, 2006; Tippo et al., 2013), all the coatings have been analyzed with FESEM (Fig. 3.3) in order to observe the surface morphology. Inserts in Fig. 3.3 display cross sections of polymer coatings, which are found to be 5-20 μm thick. The cracks and cavities observed on the PMMA-co-MAA surfaces result from the solvent evaporation during the fabrication process.

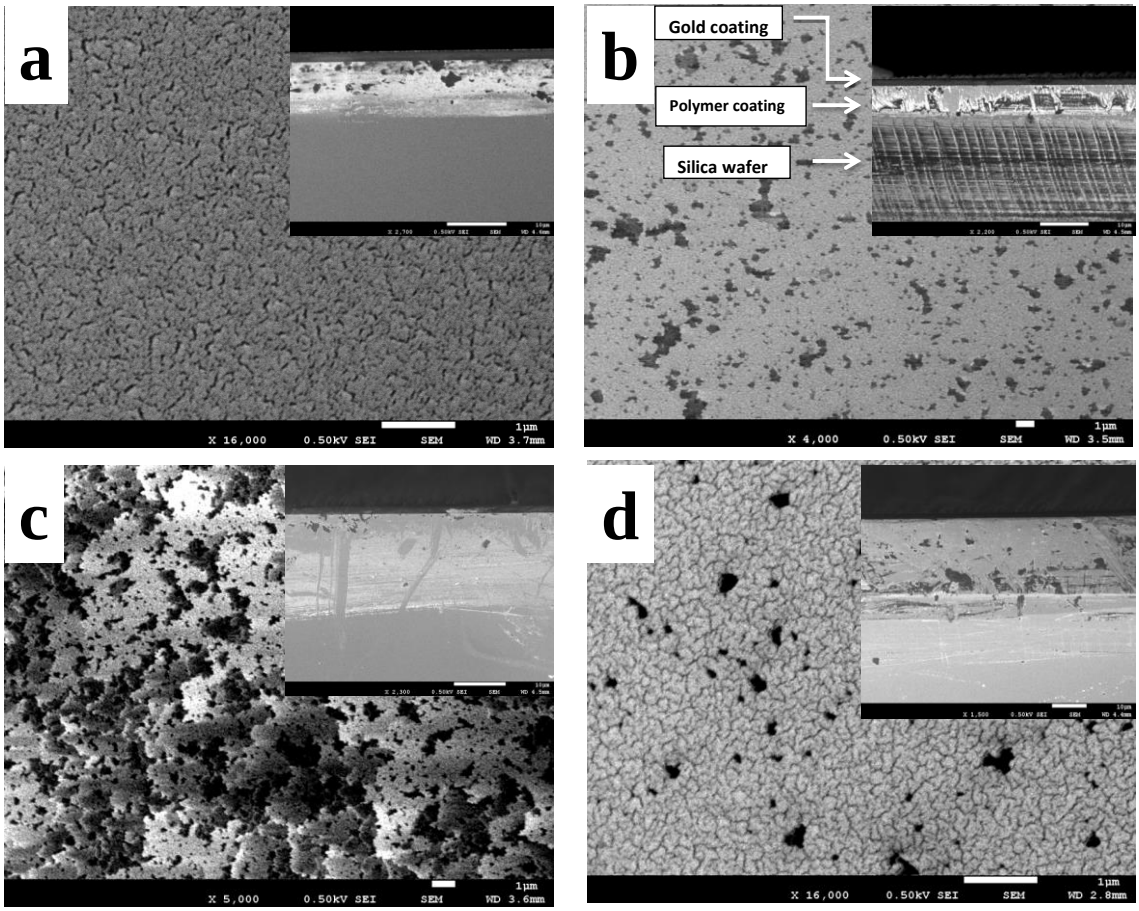


Figure 3.3: FESEM images of polymer coatings produced by spin-coating technique on silicon wafers: (a) PMMA; (b) PMMA-co-MAA (9:1); (c) PMMA-co-MAA (7:3); and (d)PMMA-co-MAA (5:5); (magnification bars = 10 µm).

The largest cavities have been observed for PMMA-co-MAA (7:3) coating displayed in Fig. 3.3c. Other PMMA-co-MAA compositions display more uniform surfaces, as observed from Fig. 3.3. Those relatively small surface irregularities could not be avoided due to the solvent evaporation in applied method (5% polymer solutions in THF have been used for spin-coating) (Dário, Macia, & Petri, 2012; Strawhecker et al., 2001). However, coatings presented in Fig. 3.3 show uniform thickness and evenly distributed surface patterns over the entire substrates used in experiments (FESEM analyzed silicon wafers: 2cm x 2cm).

The morphology of polymer coatings was characterized in AFM and the results are shown in Fig. 3.4.

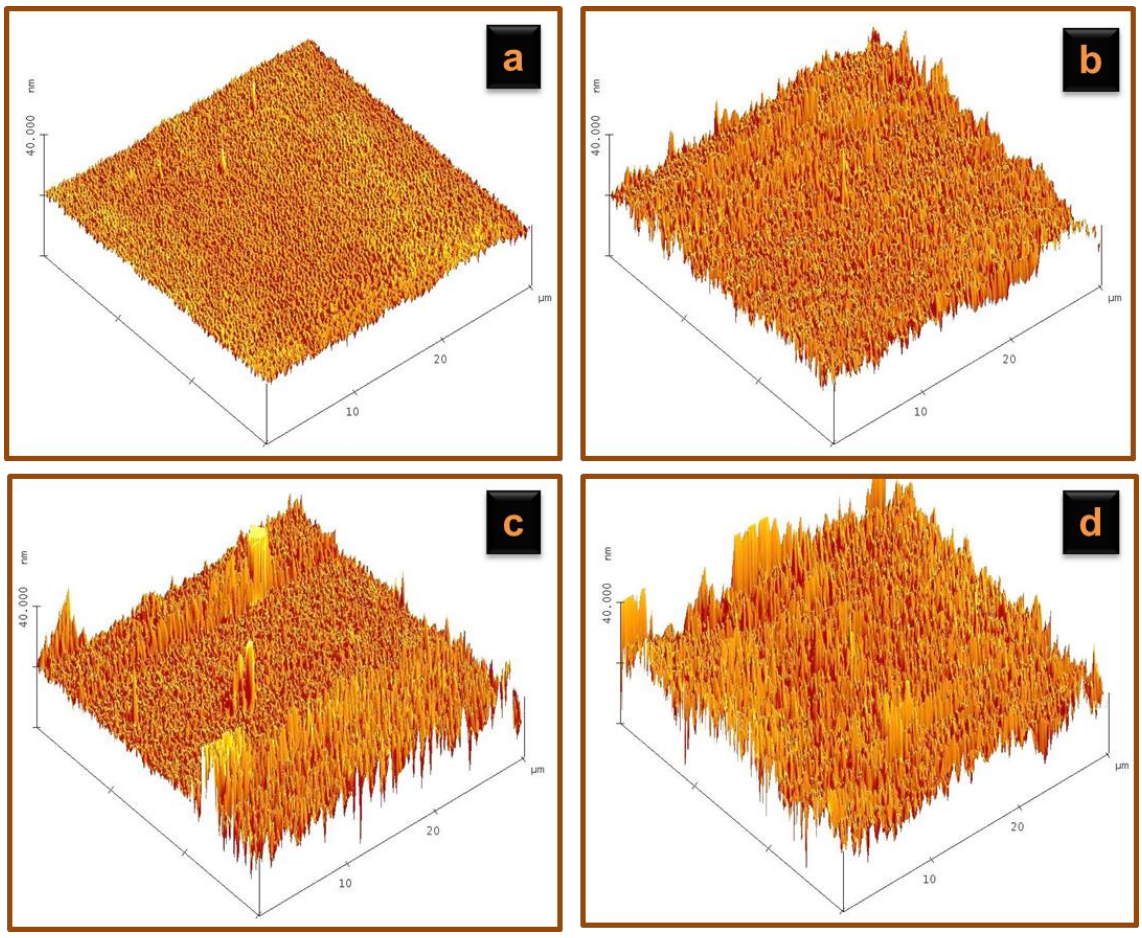


Figure 3.4: AFM topography of spin polymer coatings produced by spin-coating technique on silicon wafers: (a) PMMA; (b) PMMA-co-MAA (9:1); (c) PMMA-co-MAA (7:3); and(d) PMMA-co-MAA (5:5).

AFM images reveal both PMMA (Fig. 3.4a) and PMMA-co-MAA (9:1) (Fig. 3.4b) surfaces as smooth and uniform in comparison to the PMMA-co-MAA coatings with higher concentration of MAA groups (7:3 and 5:5; Fig. 3.4, c and d). Those particular compositions display heterogeneous morphology and increased surface roughness (Fig. 3.4c and d). The AFM results correspond with FESEM analysis (Fig. 3.3) and the increase in surface roughness was expected for the alteration in macromolecular structure (increased concentration of MAA groups in copolymer chains). The maximum mean roughness (nm) has been observed for both PMMA-co-MAA (7:3) and PMMA-co-MAA (5:5) (Table 3.2).

Table 3.2: Surface morphology data determined by AFM

Composition	Mean roughness (nm)	Max. Height (nm)	Surface area (μm^2)
PMMA	0.712	6.277	170.84
PMMA-co-MAA 9:1	1.617	20.063	153.88
PMMA-co-MAA 7:3	3.670	40.091	169.04
PMMA-co-MAA 5:5	3.358	38.889	126.39

Observed changes on surface morphology could be expected due to the hydrogel nature of the PMMA-co-MAA copolymers with substantial increase in concentration of MAA groups (Saunders, Crowther, & Vincent, 1997).

Water-in-air contact angle measurement was employed to analyze the relative surface hydrophilicity of polymer coatings (Fig. 3.5). The average contact angle for pure PMMA was measured to be 78.03 ± 0.7°, which is in agreement with previously published results (Brown et al., 2006; Meraa, Goodwin, Pike, & Wynne, 1999; Park, Lee, & Choi, 1998; Tennico et al., 2010; Zhao & Brittain, 1999).

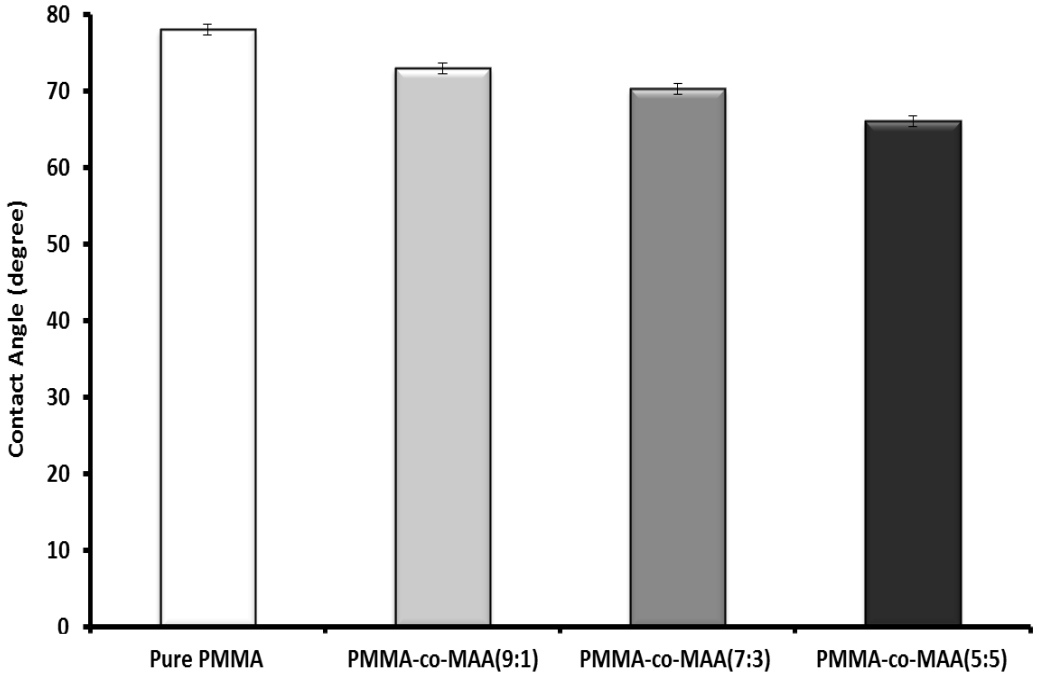


Figure 3.5: Water-in-air contact angle measured for polymer coatings.

The relative change in water contact angles from PMMA to different ratios of the copolymers (PMMA-co-MAA) clearly indicate that the chemical change had occurred at the surface due to a different molar ratio of the monomers used in polymerization. The relative increase in concentration of MAA segments induces the increased number of free –COOH present in copolymer chains. We demonstrate that those functional groups exist on material's surface and they are evidently causing increased surface hydrophilicity. As expected, the lowest value for contact angle has been measured at the surface of PMMA-co-MAA (5:5) coating (Fig. 3.5, 66.02 ± 0.5°) (Mitchell, 2011).

The presence of negatively charged –COOH groups on the surface of PMMA-co-MAA coatings has been investigated by TB assay. This pH sensitive salt (TB) contains an aromatic cation and chloride anion and the adsorption/desorption process is pH sensitive. This method has been previously reported for photometric determination of negatively charged residues at cell surface. TB dyes in aqueous solution appear blue in color with maximum absorbance at 635 nm (Sano et al., 1993; Thethi et al., 1997). In previously published work, TB has been used to determine the presence of –COOH groups on the surfaces grafted with acrylic acid (Djordjevic et al., 2010; Li et al., 2005; Sano et al., 1993). In this article, Fig. 3.6 presents the concentration of the –COOH groups calculated from the TB UV-Vis titration of spin-coated surfaces. As observed from Fig. 3.6, the TB blue also adsorbed on the PMMA coating even though this polymer does not contain any –COOH surface groups. For that reason, the results expressed in Fig. 3.6 should be interpreted only as a comparative analysis. In case of PMMA, there is an overall negative charge, accumulated around oxygen atoms (methoxyl groups) so it is expected that a TB cation should adsorb on the PMMA surface to some extent. Furthermore, an obvious increase in TB adsorption from Fig. 3.6 with a relative increase in the number of MAA copolymer segments can only be assigned to an increased concentration of surface –COOH groups.

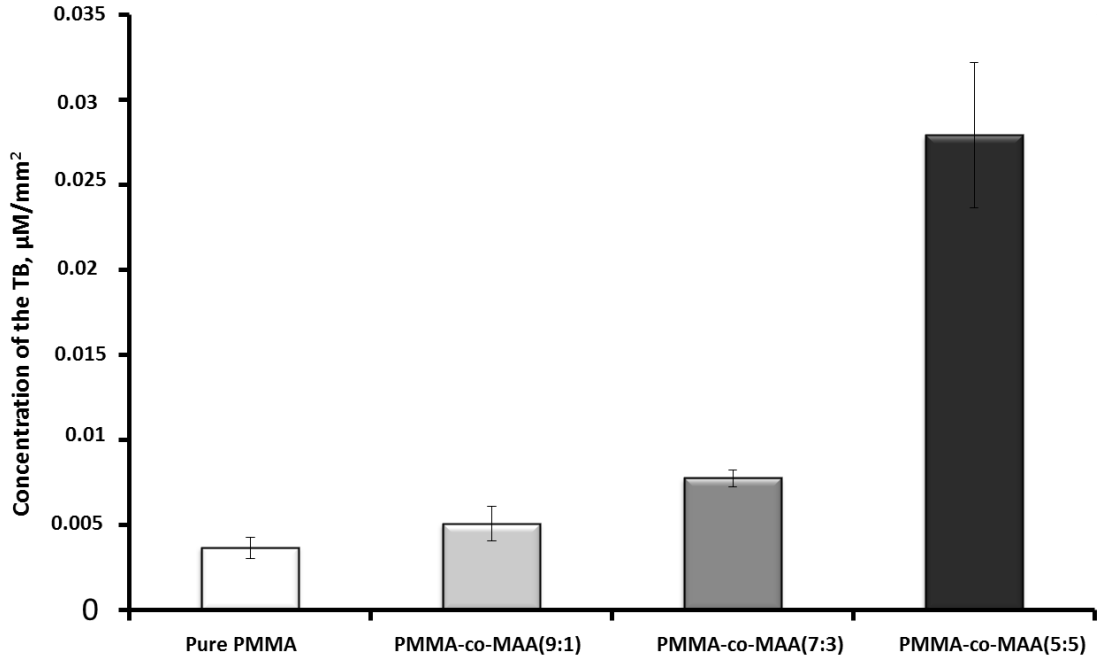


Figure 3.6: Surface concentration of –COOH groups on polymer coatings calculated from spectroscopic UV–vis surface titration with TB.

The concentration of desorbed TB from PMMA-co-MAA (5:5) coating was found to be dramatically higher in comparison to the other copolymer compositions (Fig. 3.6). Such behavior is most likely a consequence of increased polymer swelling and subsequent diffusion of TB into the bulk of the coating since the TB dye is known to complex with negatively charged groups through diffusion process (Potyrailo & Pickett, 2002). The highly hydrophilic copolymer composition PMMA-co-MAA (5:5) is expected to swell in water due to the high concentration of –COOH groups present in bulk polymer chains. B.R. Saunders et al. have previously described the behavior of PMMA-co-MAA micro-gel particles that showed a significant degree of swelling attributed to the carefully tailored polymer structures (Saunders et al., 1997). In our present case, the PMMA-co-MAA (5:5) was found to be swollen by visual observation after 2 h of TB assay. Characteristic to all hydrogel (and elastomer) systems, the loose packing of macromolecules is also reflected on mechanical properties of the material (Djordjevic et

al., 2009). Although the analysis of mechanical characteristics of PMMA-co-MAA coatings is beyond the scope of this work, it is important to note the substantial increase in surface roughness measured for both PMMA-co-MAA (5:5) and (7:3) coatings (AFM results, Fig. 3.4 and Table 3.2). Increased surface roughness must be considered in future use of PMMA-co-MAA coatings for immobilization of proteins and application in biomedical field as it offers higher contact between analyte and the substrate. This aspect is important for fundamental research as well as the application in clinical diagnostic devices. In terms of the surface chemistry, the presence of –COOH groups can be utilized for covalent immobilization of biologically active molecules. The close control over surface functionalities presents the most important advantage in that perspective. The method presented here could also provide better surface stability of the coatings since the surface functional groups are also part of the macromolecular structure designed with carefully chosen synthesis parameters.

3.5 Conclusion

In this thesis we explored a new type of PMMA-co-MAA coatings, which are potentially suitable for their use in surface protein immobilization and applications in diagnostic devices. The results from the surface analysis of three different copolymer compositions suggest that the surface micromorphology and the surface concentration of carboxyl functional groups are closely dependent on the initial concentrations of the monomers in free radical polymerization reaction. The polymer structure has been investigated with ^{1}H NMR and MALDI-ToF-MS and the relative quantification of the surface carboxyl groups has been obtained using the water-in-air contact angle measurments and TB UV-Vis assay. Our results show that the careful design of the macromolecular structure and the chemical composition of the coatings has a strong influence on the surface concentration of carboxyl functional groups. The detected surface functionalities can be further explored for physical/covalent binding of proteins

and application in various fields of biomedical science such as development of diagnostic devices, drug delivery and fundamental studies of cell-biomaterial interface.

CHAPTER 4

Structural and end-group analysis of synthetic acrylate copolymers by matrix-assisted laser desorption ionization time-of-flight mass spectrometry: distribution of pendant carboxyl groups

4.1 Introduction

A well-known strategy in the development of polymers for interaction with biological systems is control of chemically active functional groups and their concentration within polymer chains. In that perspective, there is a strong need for careful study of polymer structures and distribution of pendant functional groups within macromolecules. One of the materials of particular interest is polymethylmethacrylate-co-methacrylic acid (PMMA-co-MAA). We have performed a detailed matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-ToF-MS) characterization of PMMA-co-MAA (synthesized with different monomer ratios) in order to establish the molecular mass distribution, polymer end-groups and exact molecular structures present in the polymer systems. Experimental results have confirmed the successful formation of the copolymers, based on pre-determined theoretical compositions, with close control over macromolecular structure. Furthermore, a detailed structural analysis of each composition has provided valuable information about the variation of concentration of carboxyl functional groups, generated from MAA copolymer segments.

4.2 Literature review

Over the past two decades, mass spectrometry (MS) analysis of polymeric materials has been highly influenced by matrix-assisted laser desorption time-of-flight MS (MALDI-ToF-MS) (Byrd & McEwen, 2000). Synthetic polymers are thermally unstable and fragile when ionized by conventional methods, which has limited the use of MS as a means of characterization (Murgasova & Hercules, 2003). MALDI-ToF-MS have minimized these problems by using a soft ionization technique, which allows mass determination of

molecules by ionization and vaporization without fragmentation. Various studies in the field of molecular MS have shown that, for a wide range of polymers of limited polydispersity (PD < 1.2), MALDI-ToF-MS can provide reasonably accurate average molecular mass information (Murgasova & Hercules, 2003). MALDI-ToF-MS has the potential to provide, not only the molecular mass and mass distribution for synthesized polymers, but also a detailed and accurate end-group analysis and, in particular cases, branching information of the compounds (Wetzel, Guttman, & Girard, 2004). Taking advantages of a single fast analysis, MALDI-ToF-MS, structural information can readily be established, which is important for determination of absolute polymer structures (Byrd & McEwen, 2000).

For each successful MALDI-ToF-MS experiment, selection of appropriate matrix, solvent and ionizing agent are of a great importance. Structural analysis can be particularly crucial in the case of synthetic polymers as they vary in their polarity, solubility and behavior toward counter ions (Murgasova & Hercules, 2003). Sample preparation has also played a vital role in accuracy of the results. The final aim for matrix, ionizing agent and solvent in sample preparation is to co-crystallize the matrix and polymer in the most homogeneous way. In MALDI-ToF-MS analysis of synthetic polymers, sample preparation typically results in inhomogeneous crystallization so the “laser spots”, which give the best possible spectral resolution, have to be found manually during the experiment (Nielen, 1999). The other important parameter in successful MALDI-ToF-MS analysis is the spotting technique. Many different methods have been developed so far, such as dried-droplet method (the oldest reported method) (Karas & Hillenkamp, 1988), fast crystallization technique (by using vacuum chamber) (Carbonnelle et al., 2011; Castro & Wilkins 1992; Monrabal, 1994), electro-spray deposition (for extra homogeneous spot) (Boggio et al., 2011; Nemes, Huang, & Vertes, 2012), and spin-coated layers, which has resulted in more

homogeneous surfaces and subsequent higher sensitivity (Manier et al., 2011; Sonar et al., 2011).

Among the family of synthetic polymers, polyacrylate materials have drawn a great deal of interest in the biomedical field. Those unique polymeric materials have demonstrated excellent performance in production of biomaterials for diagnostic imaging and fabrication of biosensor devices due to their properties such as low specific weight, high impact resistance and flexibility (Hosseini et al., 2014). The general concept of using polymeric platforms in such applications relies, to a large extent, on the carful design of the polymer structure that would interface with sensitive and highly selective biological systems (Hosseini et al., 2014a). For that reason, better understanding of macromolecular composition, structure, block copolymers and end-group determination of the compounds seems to be of crucial importance. Herein, we report the synthesis and MALDI-ToF-MS characterization of polymethyl methacrylate-co-methacrylic acid (PMMA-co-MAA) polymers in different molar ratios of the monomers, methyl methacrylate/metacrylic acid (MMA/MAA), which have been prepared via the well-established method of free radical polymerization. Molecular mass determination and structural analysis of the synthesized copolymers were performed with MALDI-TOF-MS (by using layer-by-layer spotting technique). We report a detailed end-group and copolymer analysis that provides essential information, necessary for future application and design of poly acrylate systems in biomedical research.

4.3 Experimental procedure

4.3.1 Chemicals and reagents

Methyl methacrylate (MMA), methacrylic acid (MAA), 2, 5-dihydroxy benzoic acid (DHB), sodium iodide (NaI), and ethanol (EtOH) were purchased from Sigma Malaysia. Tetrahydrofuran (THF, Thermo Fisher Scientific, US) was used as solvent in polymer

synthesis and processing procedures. The free radical initiator azobisisobutyronitrile (AIBN) was purchased from Friedemann Schmidt Chemical, Germany. MMA monomer was purified by distillation before free radical polymerization. All other materials have been used as received.

4.3.2 Synthesis and processing of PMMA-co-MAA

Four different compositions of the PMMA-co-MAA were synthesized by free radical polymerization using THF as solvent and AIBN as initiator, reported previously (Hosseini et al., 2014a). In brief, MMA and MAA monomers were used in reaction mixtures with variation in initial concentrations as follows: pure PMMA, PMMA-co-MAA (9:1), PMMA-co-MAA (7:3) and PMMA-co-MAA (5:5); the numbers in brackets represent the molar ratios of MMA/MAA. In brief, a three-neck round-bottom flask was charged with 50 ml of THF and pre-calculated volume of MMA. A mixture of MAA and initiator (AIBN. 0.328 g) was added to the solution and the polymerization reaction was carried out for 6 hours at 90°C (Appendix Figures 2 and 3). The reaction was stopped by pouring the reaction solution into distilled water. The polymer precipitation was filtered and washed thoroughly with water, freeze-dried and stored in a refrigerator for further experiments.

4.3.3 Sample preparation and MALDI-ToF-MS analysis

NaI was used as ionizing agent (100 mg of NaI in 1 ml of EtOH), and matrix solution was prepared by dissolving 100 mg of DHB in 1 ml of EtOH. In order to prepare polymer solution, 10 mg of raw polymer was dissolved in 1 ml of THF. Layer by layer deposition was chosen as the spotting method. 0.5 µl of DHB solution was deposited (first layer) and dried on the sample plate. A layer of NaI solution (0.5 µl) was added on top of the dried matrix as second layer. After another drying step, the subsequent layer of the polymer solution was deposited on the top of the crystallized NaI-DHB mixture (0.5 µl) (Djordjevic et al., 2009). It is recommended to use only one solvent for dissolving ionizing agent (salt),

matrix and polymer for sample preparation. This may reduce the risk of segregation, as even relatively small concentration of polymer non-solvent might result in error in obtained data. However, salts are barely soluble in organic solvents, which can dissolve synthetic polymers (Nielen, 1999). For that reason, searching for the "sweet" spots for analysis was needed (Djordjevic et al., 2009). The sample plate was placed in the MALDI-ToF-MS (ABI 4800 plus) analyzer equipped with a nitrogen laser emitting at 375 nm and data acquisition was set to perform in positive ion mode. DATA Explorer software was used for data processing. The acceleration voltage was set to 10 kV in reflector MS mode. All MALDI-ToF mass spectra were collected by averaging the signals of at least 500 individual laser shots.

4.4 Results and discussion

One of the major advantages of MALDI-ToF-MS over other techniques is the soft ionization in which cations efficiently "wrap the polymers around themselves" (Nielen, 1999). Essentially, this method prevents fragmentation and preserves the structure of the polymer chains, thus allowing us to "see" whole molecules in the mass spectra. Species present after ionization can include a neutral molecule [M] or ionized molecular species such as: $[M+H]^+$ (added proton); $[M+Na]^+$ (added sodium ion); $[M-H]^-$ (removed proton); $[M+nNa]^+$ (more than one sodium is attached to the chain) and $[M+nH]^{n+}$ (more than one added proton) (Knochenmuss, 2006; Mochalski et al., 2014; Nielen, 1999). The possible appearance of those species makes MALDI-ToF-MS analysis complex, however, the range of the detected molecular mass peaks does not normally exceed the highest values that can be determined by other MS techniques (Byrd & McEwen, 2000; Jackson et al., 1996; Murgasova & Hercules, 2003). Figures 4.1-4.4 present MALDI-ToF mass spectra of PMMA, PMMA-co-MAA (9:1), PMMA-co-MAA (7:3) and PMMA-co-MAA (5:5), respectively. It can be observed that peaks represent the relatively low range of molecular mass (up to ~ 4000) while our previously reported result indicated the average M_W of

40000 determined by gel permeation chromatography (GPC) (Hosseini et al., 2014a). Another limitation in accuracy of macromolecular structure determination is mass discrimination (Fan et al., 2014). In most cases, mass discrimination originates from instrumental factors, such as ionization and/or transmission. In such condition, the mass detector becomes saturated by lower molecular weight fragments that cause the appearance of mass peaks with m/z values higher than calculated molecular weight for the proposed structure (Murgasova & Hercules, 2003; Nielen, 1999). Even with the molecular mass detection of relatively small macromolecules, MALDI-ToF-MS provides the unique picture of the molecular structure (Wu et al., 2013). The clear evidence of the exact distribution of monomer units (MMA/MAA) can be extracted from the spectra (Fig. 4.1-4.4), which is particularly important information for using such compounds in further complex experiments in biomedical research (Knochenmuss, 2006; Nielen, 1999).

A representative MALDI-ToF mass spectrum of PMMA is shown in Fig. 4.1. With proposed structures of $[Pol. + Na]^+$ that are shown for determined molecular weights of the polacrylate chains. The peaks at m/z=1102 correspond to the presence of 10 monomer units (MMA) and 2 Na atoms (ionizing agent) attached to the chain. The number of the monomer units in the polymer chain at m/z=2031 is 19, associated with 5 Na atoms. Consequently, m/z=3569 represents PMMA structure of 35 MMA monomer units and 2 Na associated atoms. The most favorable interaction between ionizing agent and polymer chain is expected to occur at higher molecular weight oligomers, thus a lower binding energy of ionizing agent is predictable for shorter oligomers (Nielen, 1999). For that reason, the maximum number of Na atoms, attached to the chain, can be observed from the PMMA spectrum (Fig. 4.1), which is, at the same time, the largest detected polymer chain from MALDI-ToF-MS experiment (Fig. 4.1-4.4).

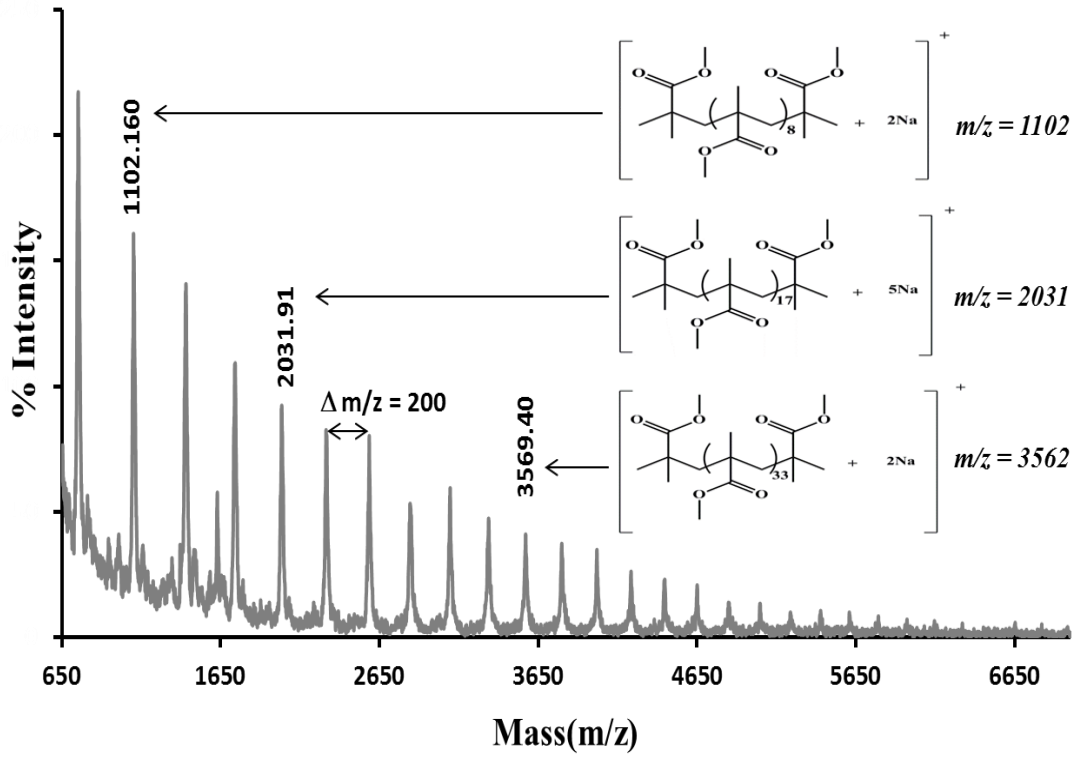


Figure 4.1: MALDI-ToF-MS spectrum of PMMA.

Note that the only monomer, which has contributed in the polymerization reaction of homopolymer PMMA is MMA, and that explains the interval between peaks (Δm/z=200), which corresponds to the molar mass of additional two MMA units in each subsequent chain.

Figure 4.2 shows MALDI-ToF mass spectrum of PMMA-co-MAA (9:1). The peak at m/z=978 presents molecular structure of the chain, which contains 4 MMA (n=4) and 6 MAA (m=6) units, in which 4 units of the MAA exist inside the chain and another 2 are placed at the end-group positions (the structure of this peak is displayed in Fig. 4.2). The peak at m/z=992 contains an equal number of MMA and MAA (n=m=5), while MAA segments have again occupied both sides of the chain (as end-groups).

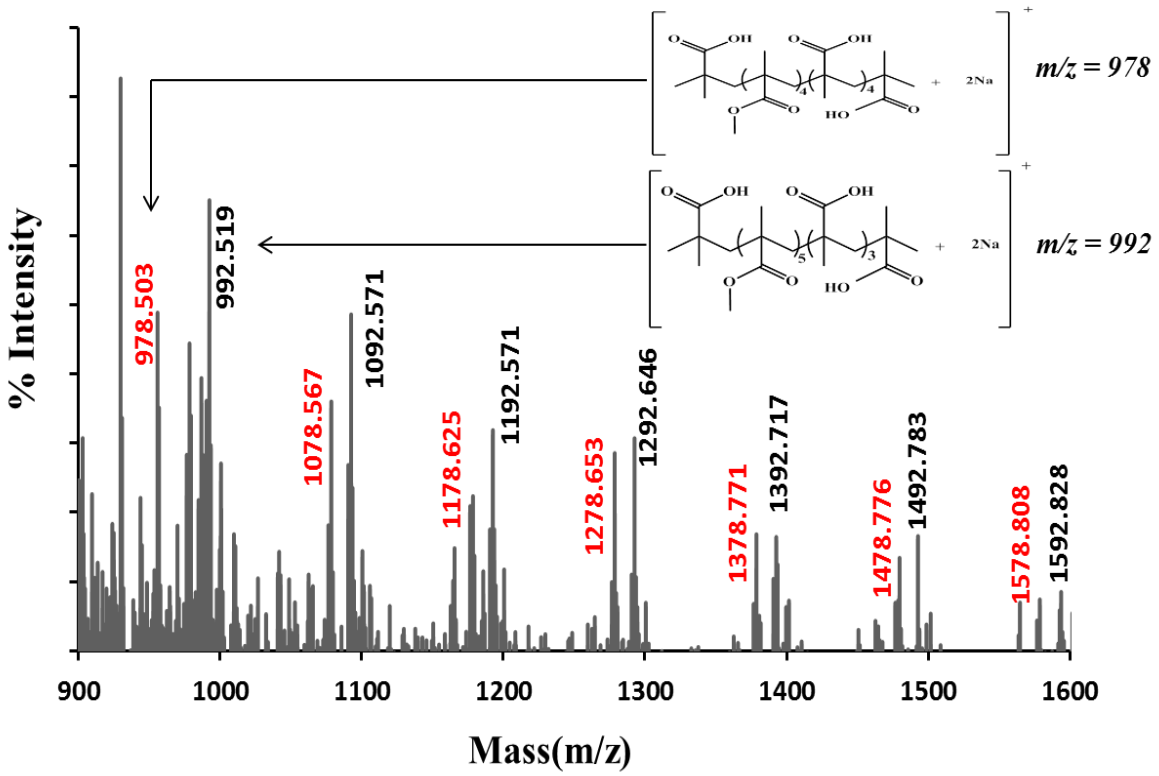


Figure 4.2: MALDI-ToF-MS spectrum of PMMA-co-MAA (9:1).

In both peaks, the attachment of 2 Na has been detected. Theoretical calculations and the proposed structures for the rest of the peaks in Fig.4.2 are presented in Table 4.1. It should be noted that Δm/z is equal to 100 for both sets of peaks (blacks and reds, Fig. 4.2), which clearly correspond to orderly increment of MMA units to the copolymer chains. This is expected as composition (9:1) offers a greater number of MMA units for chain formation in comparison to MAA concentration in the reaction mixture.

The molecular mass distribution of the PMMA-co-MAA (7:3) is shown in Fig. 4.3. Calculations suggest that, apart from the peak at m/z=725 (n=4, m=1), other peaks such as m/z=777 (n=2, m=6) and m/z=791 (n=3, m=5), have shown the increase in the number of MAA units (m). This is in accordance with pre-determined molar ratio of the copolymer (7:3) that was synthesized with initial molar percentages of 70% of MMA and 30% of MAA monomers.

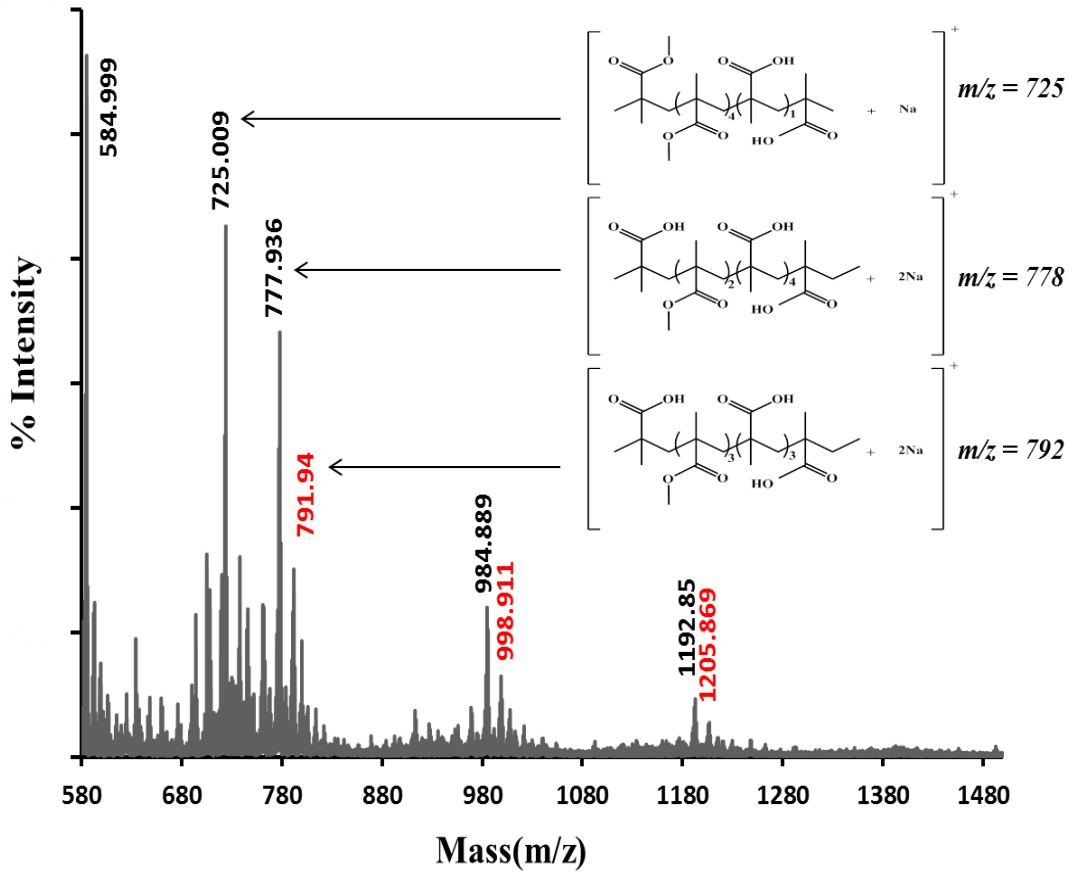


Figure 4.3: MALDI-ToF-MS spectrum of PMMA-co-MAA (7:3).

The same pattern was followed in the mass spectrum of PMMA-co-MAA (5:5) copolymer composition with higher concentration of MAA segments (Fig. 4.4). In particular, the peak at m/z=841 shows n=2 and m=7 and the peak at m/z=955 presents the possibility of having n=4 and m=6, MMA and MAA units, respectively. Similar structures with the higher number of MAA segments were calculated for the rest of the MALDI-ToF-MS peaks obtained from PMMA-co-MAA (5:5) composition (Table 4.1). This result is in very close agreement with structural analysis obtained from ^{1}H NMR spectroscopy of the same samples, where the experimental values have supported the theoretical estimation of the designed polymerization (Hosseini et al., 2014a).

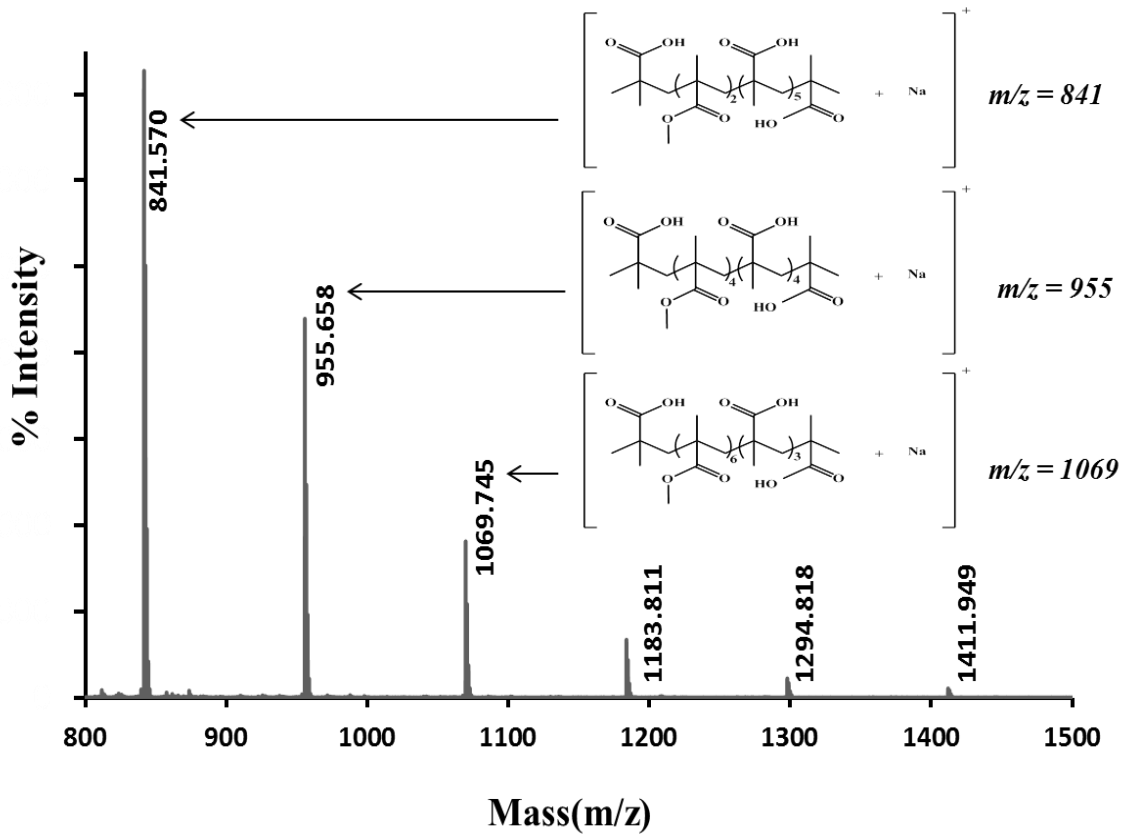
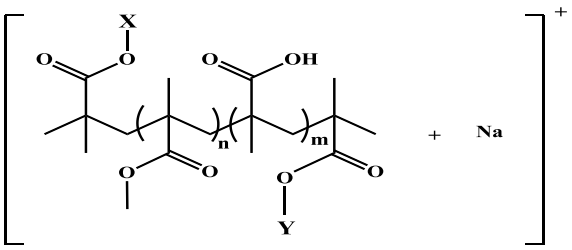


Figure 4.4: MALDI-ToF-MS spectrum of PMMA-co-MAA (5:5).

Determination of polymer end-groups provides valuable information as those groups play an important role in physical and chemical properties of the material (Tatro et al., 2003). In the case of chemical modification of the polymer chains, end-group study provides essential information as presence or absence of terminations may cause significant changes, not only in the final properties of the material, but also in reactivity of the compound (Girod et al., 2013). Table 4.1 shows the proposed structures of compounds for each detected peak, including the number of monomers as well as the experimental and calculated values for each detected peak and end-groups (X and Y can be replaced with either "H" or "CH_3" respectively).

Table 4.1: End-group analysis from detected peaks in MALDI-ToF-MS



peak position (m/z)		MMA units (n)	MAA units (m)	X	Y
Exp.	*Theor.*	*PMMA*			
1102.160	1102	8	0	CH_3	CH_3
2036.91	2037	17	0	CH_3	CH_3
3569.40	3569	33	0	CH_3	CH_3
Exp.	*Theor.*	*PMMA-co-MAA (9:1)*			
992.519	992	5	3	H	H
1078.567	1078	5	4	H	H
1092.571	1092	6	3	H	H
1178.625	1178	6	4	H	H
1192.571	1192	7	3	H	H
1278.771	1278	7	4	H	H
1292.646	1292	8	3	H	H
1378.771	1378	8	4	H	H
1392.717	1392	9	3	H	H
1478.776	1478	9	4	H	H
1492.783	1492	10	3	H	H
1578.808	1578	10	4	H	H
1592.828	1592	11	3	H	H
Exp.	*Theor.*	*PMMA-co-MAA (7:3)*			
584.999	585	1	1	CH_3	CH_3
724.009	725	4	1	CH_3	H
777.936	778	2	4	H	H
791.94	792	3	3	H	H
984.889	984	3	4	CH_3	H
998.911	999	1	8	H	H
1192.911	1192	5	5	CH_3	CH_3
1205.869	1206	6	4	CH_3	CH_3
Exp.	*Theor.*	*PMMA-co-MAA (5:5)*			
841.570	841	2	5	H	H
955.658	995	4	4	H	H
1069.754	1069	6	3	H	H
1183.811	1183	1	8	H	H
1294.818	1294	1	11	H	CH_3
1411.949	1412	1	12	H	CH_3

A gradual increase in the molar ratio of the monomer MAA can be observed from Table 4.1, which is in line with pre-calculated monomer concentrations in the polymerization reaction and the previously reported ^{1}HNMR results (Hosseini et al., 2014a).

As one of the rare examples among analyzed peaks, m/z=1069, corresponding to the spectra of PMMA-co-MAA (5:5), has revealed four different possible structures with different end-groups, which can make perfect match to the detected peak value (Figure 4.1).

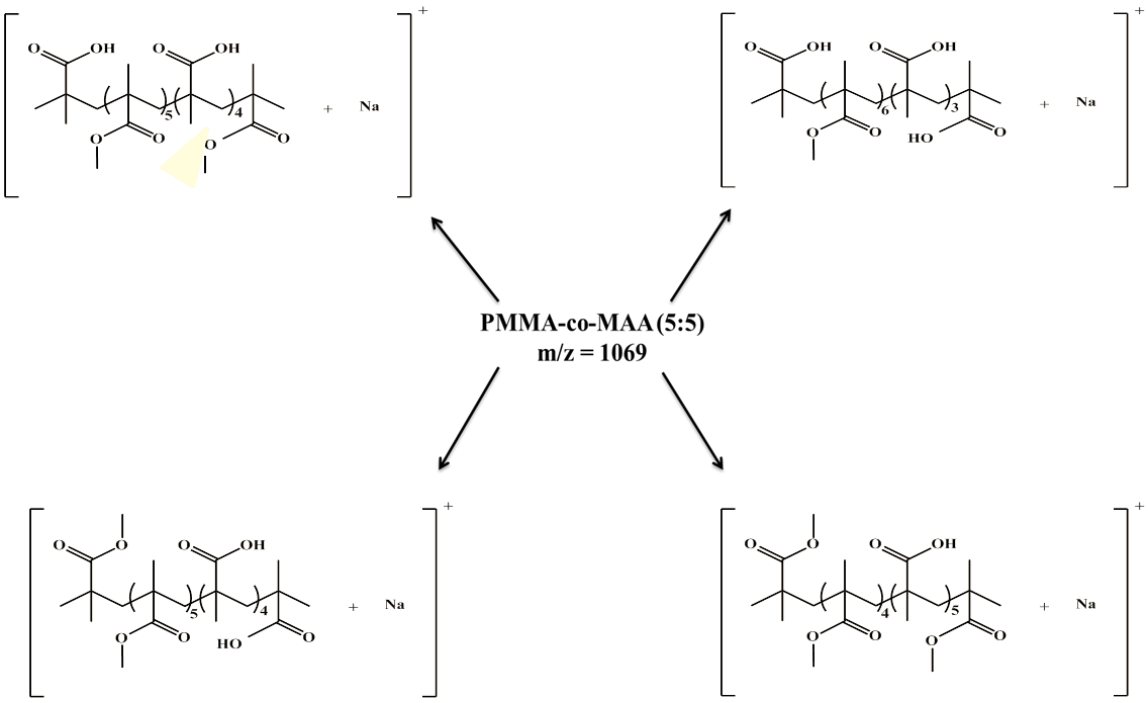


Figure 4.5: Proposed structures for the peak located at m/z=1069 in PMMA-co-MAA (5:5) spectrum.

It can be observed that, in all of the suggested structures of this specific peak, the number of monomer units is constant: n=6 (MMA) and m=5 (MAA). However, the end-groups of the copolymer can be different in each structure. This consideration shows how the end-group study by MALDI-ToF-MS can be often compromised in such cases, leading to misinterpretation of exact macromolecular structures.

Previously, we have developed PMMA-co-MAA materials composed of different MMA/MAA molar ratios, which have been designed for fabrication of a polymeric platform that can be used for covalent surface immobilization of proteins. This has been achieved with regards to the hypothesis that permanent carboxyl groups (generated from MAA segments) exist on the polymer surface. Those surface carboxyl groups, not only provide functionalities for protein immobilization (via carbodiimide chemistry), but also contribute to controlled hydrophilicity of the polyacrylate surfaces. In particular, silicon spin-coated polymer surfaces of different molar ratios of the monomers MMA/MAA were prepared with variation of surface chemistry, morphology and functionality (Hosseini et al., 2014a). Therefore, a detailed structural analysis of polymer compositions associated with other experimental work (surface analysis) provides us with a clear picture for further possibilities regarding protein activation of such surfaces. For example, the correlation between water-in-air contact angle study and MALDI-ToF-MS analysis has drawn a great deal of interest. Increasing the number of MAA segments generates carboxylic groups on the outer surface of the polymers as an inseparable part of macromolecular structure (Table 4.1). Interestingly, for the exact same reason, the contact angle measurements of these compositions has resulted in much hydrophilic surfaces as the concentration of carboxyl functional groups (originating from MAA macromolecular segments) increased in the structure of PMMA-co-MAA (from 9:1 to 5:5) (Hosseini et al., 2014a; Vesel & Mozetic, 2012). These are important features that can be closely controlled by careful design of polyacrylate macromolecules (Audouin et al., 2012; Rusmini, 2007). Again, MALDI-ToF-MS has proven to be reliable and the most accurate technique for polymer structural analysis.

4.5 Conclusions

In this chapter, we report MALDI-ToF-MS characterization of polymethylmethacrylate-co-methacrylic acid with variation of monomer concentration in synthesized copolymers.

The samples have been prepared with layer-by-layer deposition technique, and our results show accurate structural analysis and exact copolymer strucures, distributed within the polymer systems. We conclude that the results from our experiments are in good agreement with pre-calculated theoritical molar ratios in the polymerization reaction. MALDI-ToF-MS provided detailed structures of all macromolecular compositions and a clear picture regarding the presence of carboxyl functional groups and end-group placements for each copolymer composition. In particular, the presence and control over exact concentration of pendant carboxyl groups is necessary for further investigation of the polyacrylate compounds and their applications for protein surface activation in the biomedical field. Our report provides systematic description and scientific data that could be of a great interest for development of polyacrylate platforms and fundamental knowledge about their structures.

CHAPTER 5

Aging effect and antibody immobilization on –COOH exposed surfaces designed for Dengue virus detection

5.1 Introduction

Polymethylmetacrylate-co-metacrylic acid, PMMA-co-MAA coatings were produced with different initial molar ratios of monomers (MMA and MAA) in free radical polymerization reaction. Polymeric platforms were specifically designed with controlled concentration of surface-exposed carboxyl (–COOH) groups that can be used as a desirable functionality for protein immobilization. Spin-coated chips were used for antibody (Ab) immobilization in order to investigate the influence of –COOH surface concentration on Dengue virus detection efficiency in enzyme-linked immunosorbent assay (ELISA) experiment. Successful immobilization of Ab was achieved by two different techniques: (1) physical adsorption; and (2) covalent immobilization by carbodiimide coupling between the surface –COOH groups and amine functionalities of Dengue Ab molecules. Produced polymer coatings were characterized with surface spectroscopy techniques (Raman and x-ray photoelectron spectroscopy, XPS) and water-in-air contact angle (WCA) measurements. In particular, this research concentrated on the effect of aging on the availability and activity of the surface –COOH groups. For that reason, WCA and Ab immobilization (ELISA) experiments were repeated on coated biochips after 3, 6 and 9 months of storage. Results in this chapter describe the robust and sustainable functionalized polymeric platform that can be used effectively for protein activation and development of highly-sensitive biosensors.

5.2 Literature review

Enzyme-linked immunosorbent assay (ELISA) has found many applications in the field of food industry, determination of peptides, proteins, hormones and drug allergens.

Possibly the most common and important clinical application of ELISA is detection of viruses in human blood (Kirsch et al., 2013). Despite the standardization and commercialization of ELISA in clinical practice the methodology has several drawbacks, such as laborious protocol, long incubation times, lack of reproducibility and high detection limits, which create serious problems in early detection of viruses (Bai et al., 2006). Since ELISA is based on heterogeneous processes that occur on interfaces between solid platforms (ELISA plates) and protein solutions (serum), there are interesting opportunities to use engineered polymer substrates to improve the assay. Well-designed substrates would enable increased solid-liquid contact surface area (i.e. more exposure of surface-tethered antibodies) and higher binding affinity between the polymer surface and proteins (Cavalcanti et al., 2012; Song, Lee, & Rhee, 2012).

One of the major drawbacks that have relatively been ignored is the performance of polymer surfaces such as polystyrene (PS) and polymethylmetacrylate (PMMA) as ELISA substrates. Although PS and PMMA are cost-effective and suitable for mass production, both are inert materials and do not contain reactive functional groups such as hydroxyl, amines or carboxylic acids. For that reason, such commercial analytical kits do not provide a surface that is particularly promoting adsorption of proteins. In order to overcome mentioned drawbacks of conventional ELISA, research efforts have been focused on the development of functionalized polymers with high degree of control over surface properties such as chemistry and morphology (Audouin et al., 2012; Bucatariu et al., 2013; Chen et al., 2012; Yonamine et al., 2012). In that perspective, poly(acrylate) and poly(methacrylate) macromolecules have drawn a great deal of interest due to their unique properties such as low specific weight, high impact resistance, transparency and flexibility (Bai et al., 2006; Hosseini et al., 2014). Different methods of surface modifications have been applied for generation of desirable functionality on the surface of such polymers. Specifically, generation of –COOH

groups on PMMA has been reported by variety of techniques such as wet chemical treatments (Bai et al., 2006; Brown et al., 2006; Noro et al., 2013; Varma et al., 2003), UV exposure (Situma et al., 2005) and different plasma treatment techniques (Coad et al., 2013; Li et al., 2005a; Rastian et al., 2014; Tennico et al., 2010; Vesel, Elersic, & Mozetic, 2012; Vesel & Mozetic, 2012). Although mentioned methods show relative effectiveness in generation of surface –COOH groups, such functionalities do not last for a long period of time as the treated polymeric platform "relaxes" back to the untreated status. In such cases, surface functional groups re-orient themselves as they naturally tend to occupy lower energy levels, thus losing their reactivity (Hosseini et al., 2014; Hosseini et al., 2014c). This phenomenon so called "aging effect" causes serious problems in terms of polymer shelf-life and availability for diagnostic devices on clinical scale for a reasonable period of time.

As a new approach and a replacement for relatively uncertain surface modification methods that can generate desirable but not stable functional groups on PMMA polymer, we have produced different compositions of copolymers synthesized by methyl methacrylate (MMA) and methacrylic acid (MAA) in different molar ratios of monomers. With this method the presence of –COOH groups at material's surface is ascertained (Hosseini et al., 2014a; Hosseini et al., 2014c). The major aim of this paper is to address the significant concerns about aging effect of functionalized polymer surfaces, produced for diagnostic applications. Variety of monomer concentrations (MMA/MAA) in reaction mixture is the key point to achieve the optimum number of surface –COOH groups. High density of surface functional groups might cause steric hindrance between biomolecules and subsequent loss of bioactivity. This phenomenon further causes the loss of proteins' ability to recognize and bind complementary molecules from a supernatant solution. Moreover, in the presence of many surface-bound –COOH groups, multiple binding of any biomolecule to the surface is likely to

occur, leading to aberrant conformations, partial loss of recognition and capacity to bind complementary solutes (Goddard & Hotchkiss, 2007; Hosseini et al., 2014c). Similar to high surface concentration of functional groups, insufficient number of surface functionalities could also cause protein deactivation in close proximity to the polymer surface. For those reasons, the essential criteria for potential biosensor surfaces require high level of control over polymer surface properties.

In our study we have utilized protein immobilization experiments of Dengue antibody (Ab) molecules and subsequent detection of the Dengue virus (DV). Dengue infection is a widespread mosquito-borne viral disease, which initially appears with Dengue fever (DF). This virus, which is typical in tropical and subtropical areas may also result in fatal manifestations such as Dengue hemorrhagic fever (DHF) and Dengue shock syndrome (DSS) (Alcon et al., 2002; Shu et al., 2003; Gubler et al., 2002; Stevens et al., 2009; Bessoff et al., 2008). In order to produce sustainable and robust polymer surfaces that can provide high sensitivity for DV detection, we have analyzed PMMA-co-MAA coatings by different polymer and surface characterization techniques. This study demonstrates a straightforward technique for improvement of polymeric materials that could find a wide application as diagnostic devices in terms of early detection of all kinds of viruses including Dengue.

5.3 Experimental procedure

5.3.1 Chemicals and reagents

MMA, MAA, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), N-hydroxysuccinimide (NHS), bovine serum albumin (BSA), monosodium phosphate (NaH_2PO_4), Tween 20, disodium hydrogen phosphate (Na_2HPO_4) and pH buffer solutions (pH=4, 7 and 10) were purchased from Sigma Aldrich, US. MMA monomer was distilled at atmospheric pressure prior to use. The other materials were used as received. Phosphate buffer saline (PBS) was purchased from Thermo Fisher Scientific,

US. Diced silicon OFET substrates (20 mm x 20 mm) were purchased from Ossila, UK. The free radical initiator azobisisobutyronitrile (AIBN) was purchased from Friedemann Schmidt Chemical, Germany. Tetrahydrofuran (THF, Sigma Aldrich) was used as solvent in polymerization and processing procedure of coatings.

5.3.2 PMMA-co-MAA synthesis and processing

Different molar ratios of MMA/MAA have been used in free radical polymerization reaction in order to produce four compositions of PMMA-co-MAA pure PMMA, PMMA-co-MAA (9:1), PMMA-co-MAA (7:3), and PMMA-co-MAA (5:5). Detailed polymerization procedure was previously reported (Hosseini et al., 2014a). The chemical structure of the copolymer is displayed in Fig.5.1a.

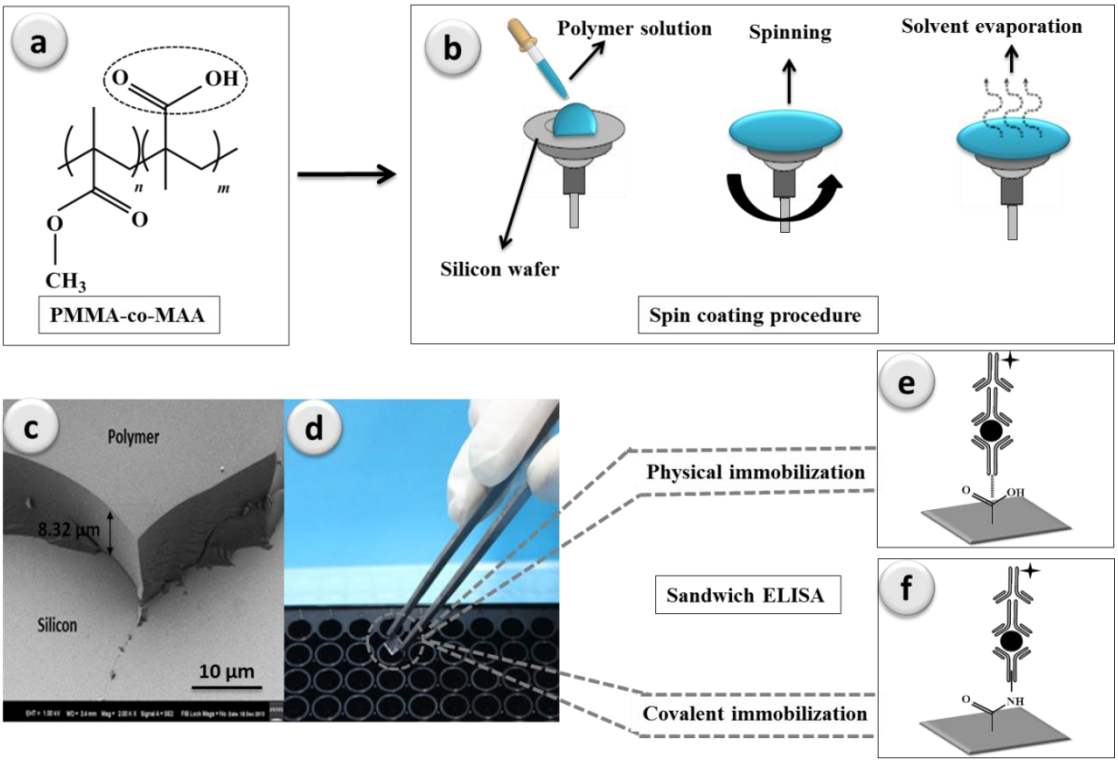


Figure 5.1: Summary of the experimental procedure: (a) PMMA-co-MAA chemical structure prepared via free radical polymerization reaction; (b) spin-coating procedure by using PMMA-co-MAA solutions of different compositions deposited on silicon wafers; (c) SEM cross-section image of the coated silicon wafer (bar = 10µm); (d) developed samples being cut into the chips with dimensions of 4 mm x 4 mm to fit 96 well-plate; (e) physical attachment of Dengue Ab to the coated surface; (f) covalent immobilization of Dengue Ab by means of carbodiimide chemistry.

For fabrication of the biochips polymer solutions of different compositions (5% polymer solutions in THF) were spin-coated on silicon wafers (20 mm × 20 mm) using spinning time of 55 seconds (3000 rpm) by Laurell, WS-650MZ-23NPP spin-coater (Fig.5.1b-d) (Hosseini et al., 2014a). Coated surfaces were cut in dimension of 4 mm x 4 mm to fit into the ELISA well-plates Fig.5.1b.

5.3.3 Polymer analysis by Raman spectroscopy

Coated surfaces of different compositions have been analyzed with Raman spectroscopy (Renishaw, model inVia). The spectral data were collected with resolution of 1 cm^{-1} using the 520 nm line of a helium–neon laser. The incident laser beam was focused on the specimen surface through a 50× objective lens, forming a laser spot of approximately 5 μm in diameter. Three coated samples of each copolymer composition have been analyzed.

5.3.4 Water-in-air contact angle (WCA) measurement

WCA of coated surfaces was measured with droplets of acidic (pH=4), neutral (pH=7) and alkaline (pH=10) buffer solutions, deposited on the solid surfaces of coated substrates. The measurement has been performed at room temperature applying sessile drop method for samples of different aging periods after coating deposition: immediate, 3, 6 and 9 months. Data physics instrument (OCA) was used for contact angle measurement. The WCA values were measured in first minutes after each droplet of water (0.1 μL) was placed on the polymer surface. Reported values are the average of five different measurements, on the center and four corners of each sample. Standard deviations were calculated for 6 samples of each polymer composition.

5.3.5 X-ray photoelectron spectroscopy (XPS)

The XPS measurements were performed by using Quantera SXMtm from Ulvac-PHI (Q1). The measurements were done using monochromatic AlKα-radiation and a take-off

angle θ of 45° at which the information depth is approximately 7 nm. A spot size of 300 × 500 μm was chosen for the sample analyses. Wide-scan measurements have identified the presence of the elements on the surface. Accurate quantification and precise identification of chemical states was performed by using narrow-scans. Standard sensitivity parameters were used to convert peak positions to atomic concentrations. Therefore, the concentrations might be deviated from reality in the absolute sense (relatively not more than 20%).

5.3.6 Dengue antibody immobilization methods on PMMA-co-MAA coatings

The coated silicon chips with the coating thickness of ~10 μm (Fig 5.1c) were cut into the dimension of 4 mm x 4 mm (Fig 5.1d) in order to fit inside the 96-well plates (SPL, life science, Korea). The total numbers of 12 samples from each composition were examined for detection of DV. Sandwich ELISA protocol was performed by using colorimetric assay in order to evaluate the efficiency of polymer coated platforms for DV detection. Apart from conventional ELISA, which was performed as a standard method (control), two sets of experiments with coated chips have been conducted: (1) physical adsorption of Ab on PMMA-co-MAA coatings (untreated surfaces, Fig 5.1e) and (2) covalent immobilization of Ab through carbodiimide chemistry (Fig 5.1f) by incubation of the chips in EDC/NHS solution (0.155 g of EDC and 0.115 g of NHS in 200 ml of PBS) for 1 hour. For covalent immobilization, samples were taken out and thoroughly washed with PBS prior to placement in 96-well plate and subsequent Ab immobilization.

5.3.7 Dengue virus propagation in mosquito cells and titration

A clinical isolate of Dengue serotype 2 from a patient's serum sample (DV2-isolate Malaysia M2, Gen Bank Toxonomy No.: 11062) was used for virus propagation by a single passage on C6/36 mosquito cells. The Dengue-infected cells with obvious

cytopathic effects (CPE) were lysed by freeze and thaw cycle. The culture medium was centrifuged at RCF=871 G for 10 min to remove cell debris, filtered (0.2 µm), portioned into aliquots, and stored at -80°C until used. The viral titer of the Dengue suspension was established by serial dilutions on Vero cells using plaque assay. In brief, a 10-fold serial dilution of medium supernatant was added to new Vero cells grown in 24-well plate (1.5×10^5 cells) and incubated for 1 h at 37 °C. The cells were then overlaid with DMEM medium containing 1.1 % methylcellulose. Viral plaques were stained with naphthol blue-black dye after 5 days of incubation. Virus titers were calculated according to the following formula: titer (p.f.u/ml) = number of plaques / volume of diluted virus added to the well × dilution factor of the virus used to infect the well in which plaques were enumerated. The titer of DV that was used in the following experiments of colorimetric ELISA was 3.5×10^7 p.f.u/ml and the serial dilutions were prepared in PBS. In order to eliminate the cross- reactions due to the host cell proteins, the cell supernatant of non-infected cells was used as a control in the ELISA assay. The values that represent a possible non-specific reaction between the host cell proteins and Dengue Ab were subtracted from the original values to increase the ELISA sensitivity.

5.3.8 Sandwich colorimetric ELISA assay on PMMA-co-MAA coatings

Each well of the ELISA well-plate was charged with 100 µl of capture Ab (Goat IgG anti DV 2 (D-15): SC-325014, Santa Cruz), which was diluted (1 : 300) in coating buffer (0.85 g of NaCl, 0.14 g of Na_2HPO_4 and 0.02g of NaH_2PO_4 in 100 ml of PBS). Incubation was carried on for 2 hours in 37 °C. Washing step was performed with 200 µl per well of washing buffer (0.05 % Tween 20 in PBS) at room temperature. ELISA kits of both, empty and including polymer chips were washed three times (each time 5 minutes) by using shaker with shaking speed of 1000 rpm. The exact same washing procedure was performed between each two steps of the ELISA. In order to achieve high selectivity and avoid non-specific bindings, blocking procedure was conducted by

adding 100 µl of blocking buffer (1 g of BSA in 100 ml of washing buffer) to each well (37 °C for 1 hour). Dengue enveloped virus was diluted (serial dilution) in variety of concentrations in coating buffer. Depending on the application, different concentrations of the virus have been used in the assay. Different concentrations in the range between 3.5×10^{-6} p.f.u/ml to 3.5×10^{-2} p.f.u/ml were chosen for determination of the calibration curves (by using Log scale). Detection range was studied by performing sandwich ELISA assay in the concentration range of 3.5×10^{3} p.f.u/ml to 3.5×10^{-6} p.f.u/ml. In particular, concentration of 3.5×10^{-2} p.f.u/ml was chosen for detection comparison as using this DV concentration has resulted in the highest detection signal. Each well was charged with 100 µl of the virus solution and incubation was carried out overnight in 4°C. Primary Ab solution was prepared by diluting mouse IgG anti DV (ICL2: SC-65725, Santa Cruz) (1:300) in diluting buffer (0.4 g BSA, 4 ml PBS buffer and 120 µl of Trintonx-100 in 36 ml of distilled water). Each well has received 100 µl of Ab solution and ELISA kits were placed in incubator for 2 hours at 37 °C. For accuracy of the judgment, chips were taken out from the analytical kit, washed and placed in the new ELISA kit prior to further steps of experiment. The incubation of 30 minutes was done at 37 °C by adding 100 µl of anti-mouse IgG (Fc specific) as secondary Ab (A-11001, Alexa Flour), which was diluted (1:200) in diluting buffer. Wells were thoroughly washed (as it was described) and filled with 100 µl of mixed substrate (Alkaline phosphatase blue micro-well substrate components A and B). Eventually reaction was stopped after 10-15 minutes by adding 50 µl of alkaline phosphatase stop solution (A585, Sigma) and signal intensity was recorded by using Bio-Rad (model 680) at the wavelength of 570 nm. The ELISA detection results were plotted after subtraction of the cut-off values from the raw data obtained from the assay. Only those samples that resulted in optical density (OD) above cut-off values (calculated as twice of the mean values for negative controls) were considered as positives (Alcon et al.,

2002). Negative controls were measured as a result of running the assay in the absence of antigen (n=8). Positive detection results were investigated as the average value of 12 replicates (n=12). It is also noteworthy that the risk of cross reactivity was less than 2%. Total numbers of 512 samples have been used in sandwich ELISA to assess the potential of the developed platforms in biological analysis. Precise calculations of sensitivity and specificity were defined by 384 positive samples with predetermined DV concentration and 128 negative controls where utterly non-infected samples were analyzed. Moreover, the accuracy of the assay has also been calculated from negative and positive readings (true and false) in comparison to the total number of the actual samples, which have been examined. Limit of detection (LoD) for each separate platform was calculated from the slopes of the calibration curves and standard deviations (Shrivastava & Gupta, 2011).

5.4 Results and discussion

5.4.1 Polymer analysis by Raman spectroscopy

Copolymers of different compositions have been analyzed by Raman spectroscopy and results are shown in Fig 5.2. Detected wavelengths of the most dominant bands in spectra are marked in the Figure. The peak in the range of 2800-3000 cm^{-1} is the most prominent since it originates from combination of different types of C–H stretching vibrations (Dybal & Krimm, 1990; Thomas et al., 2008). This spectral band consists of three overlapping peaks, which agrees well with data from the literature (Dybal & Krimm, 1990; Thomas et al., 2008). In brief, this combination band includes vibrations such as ν (C–H) of O–CH_3, α-CH_3 and –CH_2 (Thomas et al., 2008).

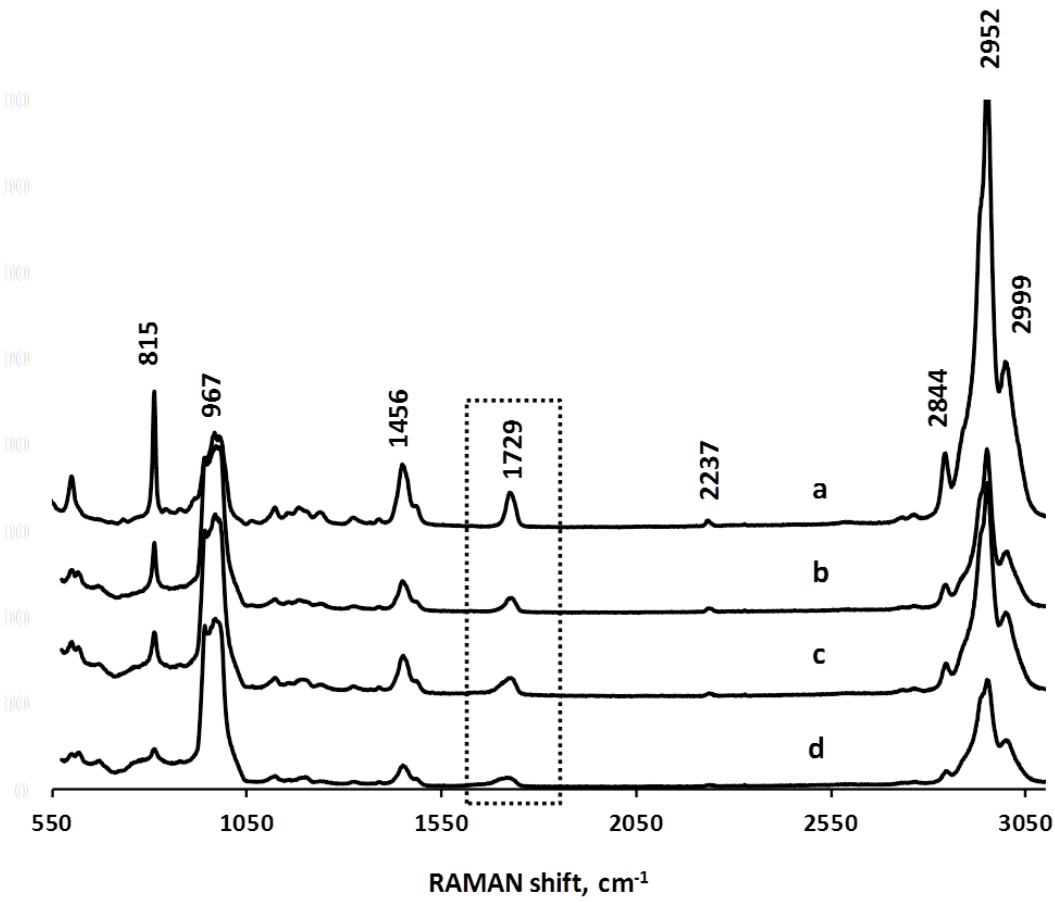


Figure 5.2: Raman spectra of produced polymer coatings: (a) PMMA; (b) PMMA-co-MAA (9:1); (c) PMMA-co-MAA (7:3); (d) PMMA-co-MAA (5:5).

Results in Fig 5.2 show systematic decrease in intensity, which is attributed to decrease in MMA content upon going from PMMA to PMMA-co-MAA (5:5) (Fig 5.2b-d). Other peaks also showed the similar pattern of decrease in intensity (bands at 815 and 1456 cm^{-1}) due to systematic replacement of –COOCH3 with –COOH groups, generated from MMA and MAA, respectively. Carbonyl (C=O) band detected at 1729 cm^{-1} for PMMA is a sharp peak in comparison to broad bands at the same wavelength, detected for other PMMA-co-MAA coatings (highlighted section, Fig 5.2) (Dong & Ozaki, 1997; Nge et al., 2002; Schilli et al., 2004). The exposed –COOH groups engaged in hydrogen bonding, cause the appearance of a broad band in the same spectral position (Dong, Ozaki, & Nakashima, 1997; Nge et al., 2002). This is a well-known phenomenon, which

clearly shows the presence of pendant –COOH groups in developed polymer systems. The concentration of –COOH is controlled by tuning the polymerization reaction parameters; in current case this is the variation of MMA/MAA initial molar ratio in reaction mixture. Inherent –COOH groups (generated from MAA monomers) are also expected to be exposed at the outmost surface layer of the coatings. All our samples demonstrated high level of consistency in terms of coating thickness and surface morphology (Fig 5.1) as reported previously (Hosseini et al., 2014a; Hosseini et al., 2014c).

5.4.2 Surface analysis with XPS

The chemical composition at the surface of the four copolymer structures of the copolymer was analyzed with XPS and results are displayed in Table 5.1. Peak positions in eV and the chemical assignments are indicated in the top row ("-"denotes that the concentration is less than the detection limit: < 0.1 %). Results demonstrated a decrease for the C1s peak (286.4 eV), which is attributed to carbon atoms in the –O–CH3 groups (typical for the MMA building blocks). Logically, the intensity of this signal decreases upon increasing the concentration of MAA segments from PMMA to PMMA-co-MAA (5:5), (Table 5.1). This is naturally causing an increase in –COOH group's concentration on the surface of PMMA-co-MAA coatings. An obvious increase in aliphatic carbon concentration (detected at 284.8 eV) can be observed from Table 5.1 as the concentration of MAA increases. This result could be predicted as the number of aliphatic carbons gradually increases in comparison to the total number of carbon atoms in PMMA-co-MAA chains. However, it can be seen from Table 5.1 that the values for O-C=O peak (288.7 eV) and O 1s peak (530 eV) have not shown considerable changes.

Table 5.1: Surface concentrations (in %) of the investigated PMMA-co-MAA samples by XPS (small concentrations of Na, K, Cl and some organic Si were neglected in presented data).

Peak		**C 1s**		**N1s**	**O1s**
Binding energy (eV)	284.8	286.4	288.7	400	530
Peak assignment	**-CH**	**-CO**	**O-C=O**		
PMMA (%)	42	16	12	0.2	26.5
PMMA-co-MAA (9:1)	44	15	13	0.5	26
PMMA-co-MAA (7:3)	45	13	12.5	0.1	27
PMMA-co-MAA (5:5)	48	10.5	11.5	0.2	26.5

A very small amount of nitrogen (400 eV) on the surface was also recorded, which is a consequence of unavoidable surface contamination and sensitivity of XPS technique. In general, XPS analysis confirms that the content of MAA increases upon changing the polymer structure in ordered form. The –COOH groups do exist on the outermost surface layer of PMMA-co-MAA coatings and their concentration gradually increases with higher concentration of MAA segments in polymer structures (Hosseini et al., 2014a; Hosseini et al., 2014c).

5.4.3 Contact angle analysis and aging effect

WCA experiment was conducted in order to determine the relative surface hydrophilicity of polymer coatings. Furthermore, the experiment was repeated on the exact same coated samples in different shelf-life periods (3, 6, and 9 months) in order to analyze the possibility of aging effect (Vesel & Mozetic, 2012). As a control and for the accuracy of the method, WCA was measured for pure PMMA as well. The results have shown the WCA measured in the range of 72-82° for PMMA, which is in agreement with previously reported results (Brown et al., 2006; Tennico et al., 2010). Gradual changes in WCA from PMMA to different molar ratios of PMMA-co-MAA copolymers

clearly indicates that chemical changes had occurred on the surface due to the different concentrations of monomers used in polymerization reaction (Fig 5.3). The relative increase in MAA segments has resulted in more hydrophilic surfaces, as a consequence of –COOH surface groups' exposure (Fig 5.4) (Hosseini et al., 2014a).

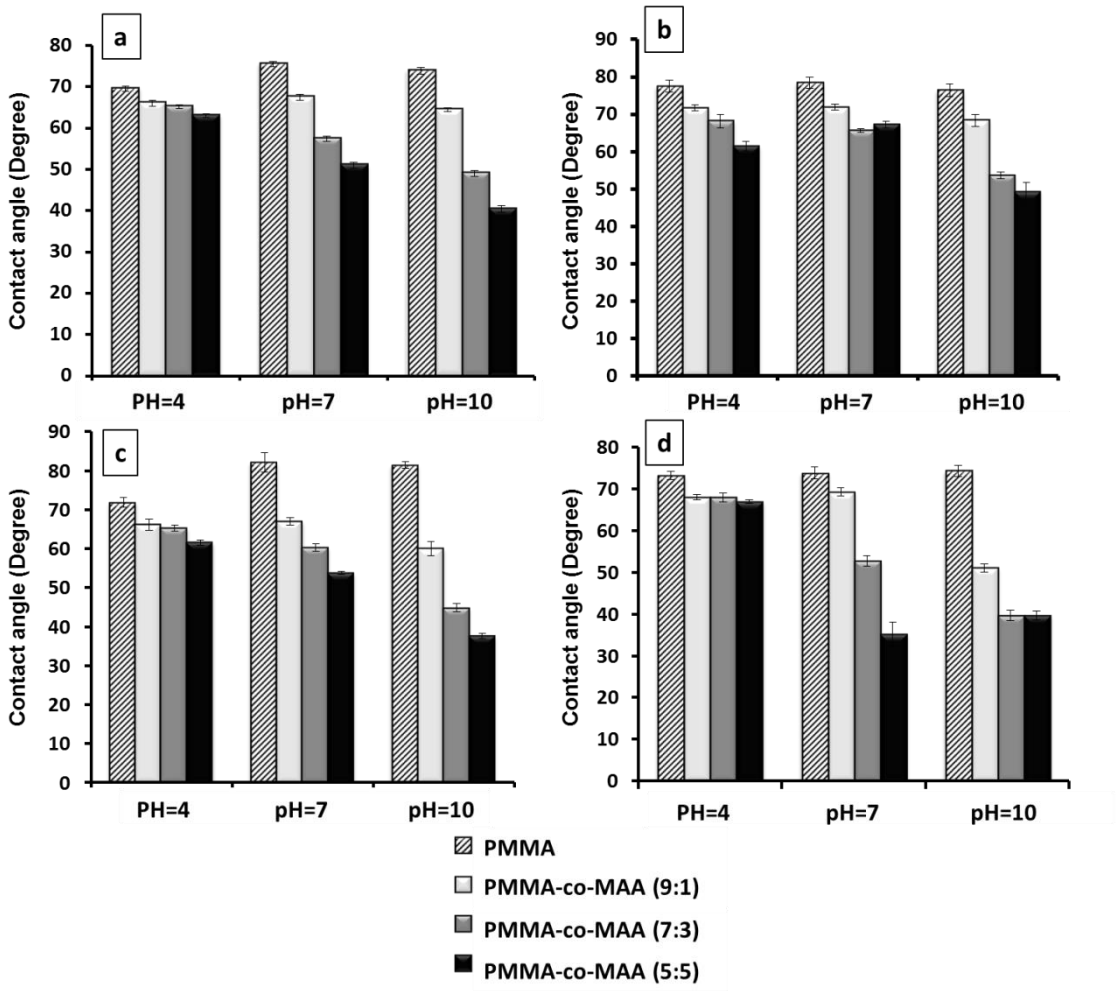


Figure 5.3: Influence of pH on water-in-air contact angle measured for PMMA-co-MAA coatings after different aging periods: (a) immediate coatings; (b) 3 months; (c) 6 months; (d) 9 months.

As expected, the lowest contact angles have been measured at the surfaces of PMMA-co-MAA (5:5) regardless of the age of the samples. In order to study the response of the coated surfaces to different environments, detailed behaviour of the samples have been

analyzed by contact angle measurements, but in a slightly different manner as usual. By depositing droplets of different mediums such as pH=4 (acidic), pH=7 (neutral) and pH=10 (alkaline), the interaction between coated surfaces and droplets have been carefully studied (Fig 5.3 and 5.4).

In the case of acidic environment (pH=4), and assuming that the –COOH groups at the surface have a dissociation rate comparable to acetic acid (pKa=4.76), it can be readily calculated that approximately 85% of the surface acid groups might not be dissociated (i.e., neutral, –COOH), while approximately 15% of them can be dissociated (i.e., negatively charged, $–COO^-$). Hence, it was predicted that the PMMA-co-MAA surfaces exhibit hydrophobic properties (relatively high WCA) at pH=4.

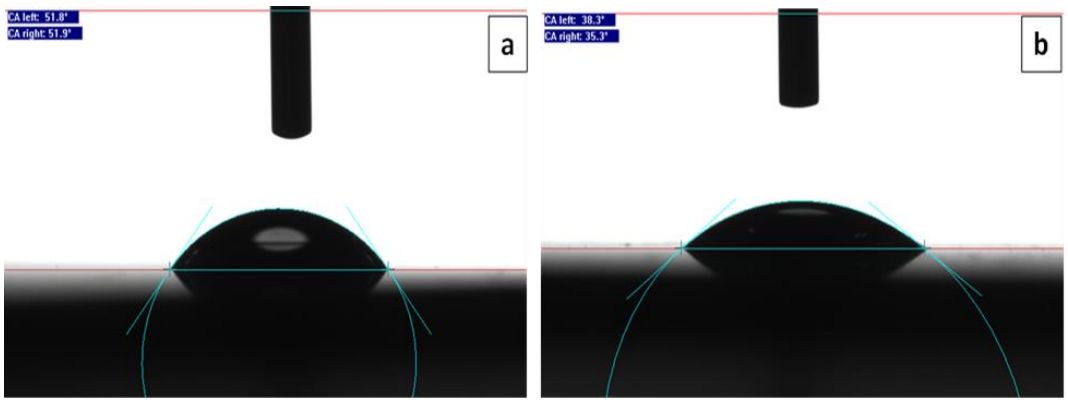


Figure 5.4: Representative picture of water-in-air contact angle and the influence of pH on PMMA-co-MAA (7:3) coating, 6 months after preparation: (a) pH=7; (b) pH=10.

In the case of a basic environment however, the calculated percentage of dissociated acid groups exceeds to 99.999. Following the same logic, hydrophilic behaviour of the surfaces (relatively low WCA) are quite understandable due to the multiple negatively charged $–COO^-$ groups at pH=10. Such a dramatic change in WCA with pH is not expected to occur in the case of PMMA as there are no –COOH groups present on this particular polymer (Fig 5.3). Data in Fig 5.3, nicely confirm these hypotheses as well.

The gradual decrease in contact angle upon going from pH=4 to pH=7 and pH=10 reaches to the lowest possible angles for PMMA-co-MAA (5:5), which is quite concentrated with –COOH functionalities (Schmidt et al., 2010). Such a simple but essential physical characterization provides unequivocal evidence for presence of –COOH groups at the surface of copolymer compositions. More importantly, the behaviour of the surfaces remains almost the same over different periods of time. Therefore it can be concluded that the strong presence of –COOH functional groups would not be affected by time. This fact, to a large extent, overcomes the major concern about aging effect, recalling the fact that instead of applying relatively uncertain techniques for generation of desirable functional groups, a tuneable functionalized platform can be chemically synthesized.

5.4.4 Dengue virus detection in ELISA

5.4.4.1 Evaluation of analytical method

Chips of different PMMA-co-MAA compositions have been examined for detection of DV through direct attachment of Dengue Ab to the surface and covalent surface immobilization of the biomolecules. Calibration curves for different coated platforms have been plotted (Fig 5.5) by running the assay in different concentrations of DV from 3.5×10^{6} to 3.5×10^{-2} p.f.u/ml. Conventional ELISA has also been conducted in commercial polystyrene kits as a control. It can be seen from Fig 5.5 that the average R^2 value of the curves resulted from polymer coated surfaces (R^2 ~0.98) are higher than conventional ELISA (R^2~0.83). Results obtained from this analysis suggest that the proposed platforms (coatings) offer more reliability in DV detection. A concentration range between 3.5×10^{3} to 3.5×10^{-6} p.f.u/ml was chosen to evaluate the detection range of the developed platforms in comparison to commercial assay (Fig 5.6). Physical immobilization of Dengue Ab to the surface in sandwich ELISA was performed for this experiment. Interestingly resulted detection signal increased as DV concentrations

decreased. This increase in signal intensity, however, was limited to the certain DV concentration (3.5×10^{-2} p.f.u/ml).

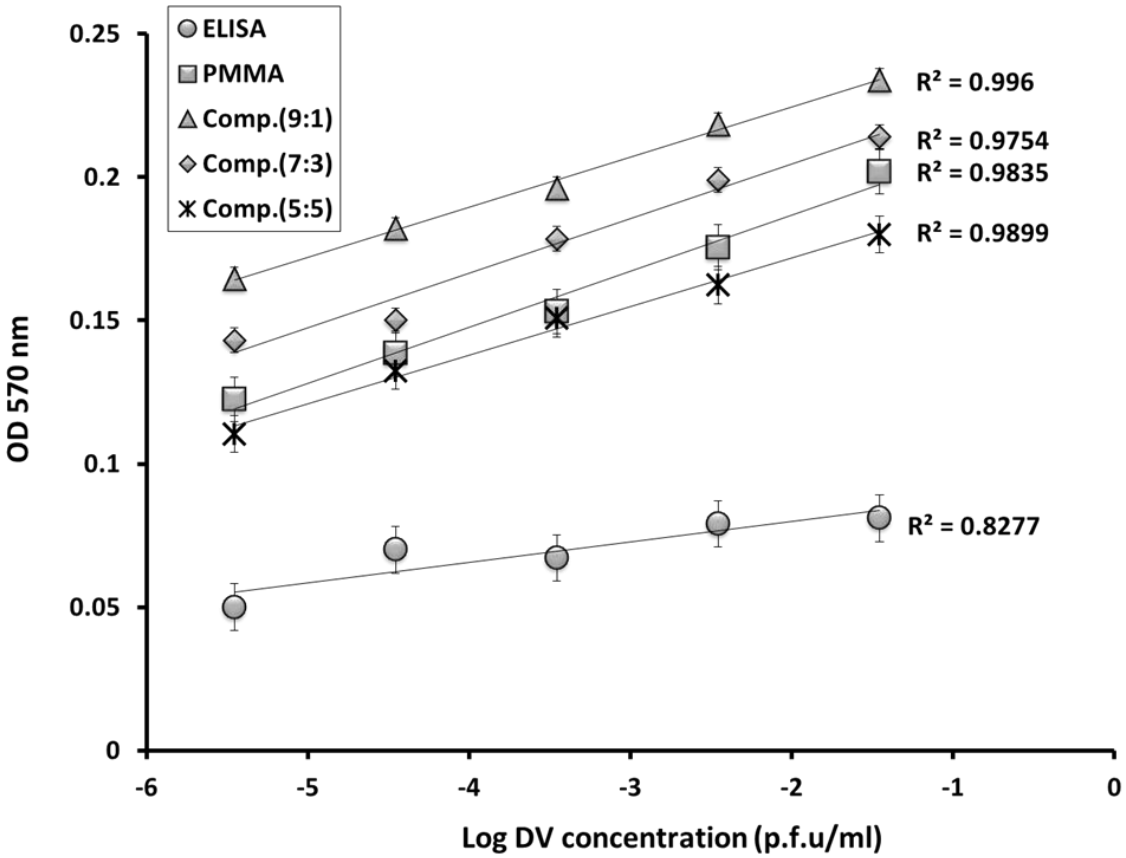


Figure 5.5: Sandwich ELISA calibration curves obtained from coated samples of different polymer compositions with acceptable level of confidence (average 98%) and conventional ELISA assay (~83%). Numbers in legend present different molar ratios of PMMA-co-MAA.

This is most likely a result of a large size of Dengue Ab molecules, which could lose their activity by denaturation caused by steric hindrance. For that reason, lower concentration of such molecules can interact with the surface in a more efficient manner. Efficiency of the assay gradually decreased as DV concentration decreased to the lowest examined levels (3.5×10^{-6} p.f.u/ml).

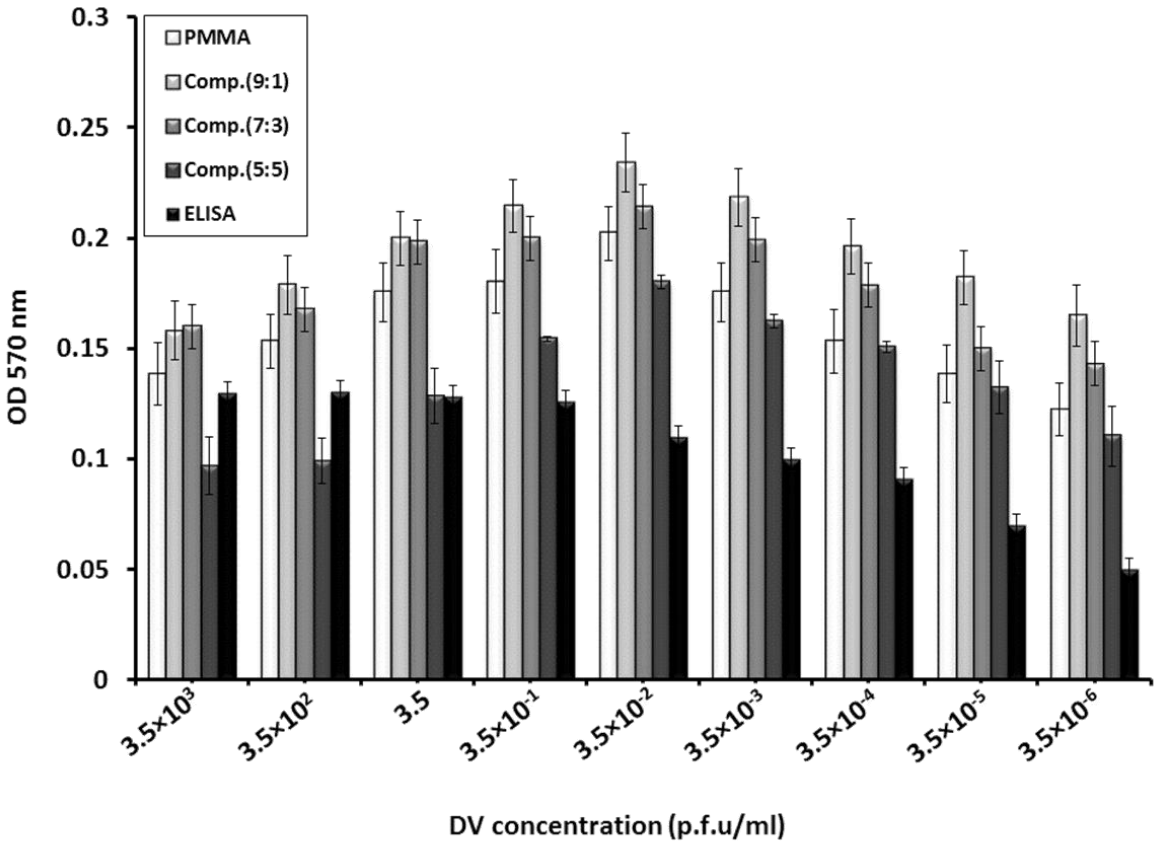


Figure 5.6: Detection range analysis performed on immediate polymer coated surface (as representative) via physical immobilization and conventional ELISA (polystyrene) in a broad range of DV concentrations.

Nevertheless, presented results clarify that mentioned bioreceptor surface is adequate to be used in diagnostics as the signal received from the platform remained positive even in the lowest concentrations of the DV. Note that all the presented results have been plotted after subtraction of the cut-off values from the original data. Sensitivity and specificity of the proposed methodology were calculated and presented in Table 5.2. It is known that non-specific bindings at intentional controls produces background signals, which can be quantitatively compared to the obtained results and establish the limits (Linares et al., 2013). Satisfactory results have been achieved by this comparison showing above 90 % sensitivity for coated samples of all different compositions.

Table 5.2: Detection sensitivity, specificity and accuracy determination by using the total number of 512 samples (384 positive and 128 negative); limit of detection (LoD) values were calculated from the slopes of calibration curves and standard deviations**.**

DV status	PMMA		PMMA-co-MAA (9:1)		PMMA-co-MAA (7:3)		PMMA-co-MAA (5:5)	
	+	-	+	-	+	-	+	-
Positive	88	3	94	2	91	1	89	2
Negative	8	29	2	30	5	31	7	30
Total	96	32	96	32	96	32	96	32
Sensitivity (%)	91.67		97.92		94.79		92.71	
Specificity (%)	90.62		93.75		96.88		93.75	
Accuracy (%)	91.4		96.87		95.31		92.96	
LoD (p.f.u×10^3/ml)	16.7		1.22		0.963		41.21	

Highest sensitivity (97.92 %) has been achieved from PMMA-co-MAA (9:1) coated samples while the highest specificity (96.88 %) was obtained from coated samples with composition PMMA-co-MAA (7:3). Furthermore, the accuracy of the assay was determined and results are presented in Table 5.2. The acceptable level of accuracy was achieved from coated surfaces of different compositions as well. The highest level of accuracy (96.87 %) was reached by using the coated platforms from comp.(9:1).

The LoD values have been calculated for coated biochips of all different compositions and results are shown in Table 5.2. All of the purposed platforms successfully detected DV in the acceptable concentration range reported for Dengue patients (day 4 to 6 of the infection) (Thomas et al., 2010). Coated surfaces with comp.(7:3), in particular, have shown the capability of the detection in the lowest DV concentrations (0.963 ×10^3 p.f.u/ml) among all the tested platforms.

5.4.4.2 Aging effect on Ab surface immobilization and reproducibility of the method

Figure 5.7 presents a detailed comparison of the performance between PMMA-co-MAA compositions with different aging periods in detection of DV. The Ab immobilization was performed by both techniques, physical and covalent immobilization and the virus concentration was chosen to be 3.5×10^{-2} p.f.u/ml (taken from the results presented in Fig 5.6).

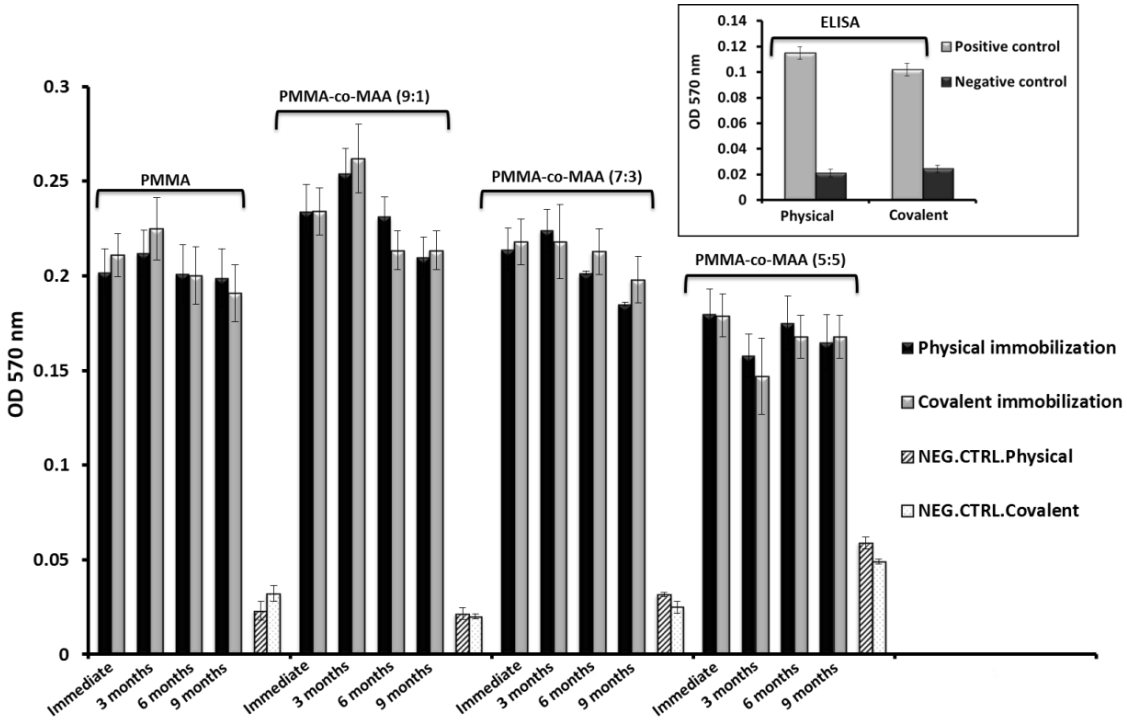


Figure 5.7: Performance comparison between coated samples of all PMMA-co-MAA compositions with different aging periods in sandwich ELISA assay aimed for DV detection (DV concentration=3.5×10^{-2} p.f.u/ml); insert: conventional ELISA, which was conducted under the same reaction conditions and the negative control (recorded signal in the absence of the virus).

Negative controls, which were conducted in the absence of DV were also measured for all of the different compositions of coated samples with different aging. However, it

was observed that the negative controls of aged samples have shown ignorable difference with the immediate coated samples (data not shown). Therefore the negative controls in Fig 5.7 (determined from physical and/or covalent immobilization) are representatives for all of the age categories from immediate coated to maximum 9 months old surfaces. Again, it is important to note that signal intensities in Fig 5.7 were plotted excluding negative controls. Conventional ELISA (insert in Fig 5.7) was performed as control with the exact same DV concentration (3.5×10^{-2} p.f.u/ml) along with its negative controls resulted from, both, physical and covalent immobilization. Aged samples of 3, 6 and 9 months old were used in ELISA assay under the same reaction conditions as immediate coatings (Fig 5.7). Same trend as immediate coated surfaces was observed from other age categories. Although oldest samples (9 months) have resulted in the slightly lower signal intensity, higher level of detection signal in comparison to what was obtained from conventional ELISA cannot be ignored. Therefore it can be concluded that proposed surfaces, in general, have proven to be reliable and capable platforms for biosensing applications even after 9 months of the production. Although performed study reveals very fundamental aspects in surface engineering, it can effectively motivate new research areas dealing with fabrication of bioreceptor surfaces with longer shelf-life.

5.4.4.3 Comparative study of protein immobilization on PMMA-co-MAA coatings: the surface chemistry aspect

The general observation from our results presented in Fig 5.7 is that the immobilization of Ab on polymer coatings has resulted in consistently higher detection signals (regardless of the background noise) in comparison to conventional ELISA experiment. In particular, the simplified method of physical adsorption is generally reproducible and provides acceptable level of consistency in each set of the experiments. It can be clearly seen that the presence of polymer coatings has shown a great impact on the detection

signal intensity, of which (9:1) composition (with minimal concentration of –COOH groups) consistently resulted in the highest efficiency of Ab immobilization. Since oxygen dominant PMMA and PMMA-co-MAA of different compositions are rich with electronegativity, macromolecular surfaces can possibly bind to $–NH_2$ functionalities of the proteins by ionic attraction as a function of surrounding pH. However, among different forces that influence protein immobilization, ionic attraction might be less effective in comparison to other forces at the interface, such as hydrophobic interaction and hydrogen bonding (Yoon et al., 1996). PMMA with the highest contact angle among all the proposed coatings (Fig 5.3) takes the advantage of the hydrophobicity in its detection performance. Nevertheless, PMMA-co-MAA (9:1) has resulted in relatively higher detection signal even though this particular composition is not as hydrophobic as PMMA.

Yoon et al in their research work concluded that among these major forces, which can influence protein immobilization, hydrogen bonding offers a better chance of biomolecular attachment than hydrophobic interaction (Yoon et al., 1996). Aligned with their finding, our results presented in Fig 5.7 confirm the stronger effect of hydrogen bonding on protein immobilization than hydrophobic interaction. Although PMMA-co-MAA (9:1) is less privileged with hydrophobicity, surface –COOH groups generated by MAA segments (Fig 5.1a) can interact with amine groups of proteins through hydrogen binding as well, thus causing further enhancement in immobilization and subsequent detection (Ibrahim, Nada, & Kamal, 2005). This fact, to a large extent, emphasizes the crucial role of surface –COOH groups and their interaction with proteins. Interface forces are of fundamental importance for improved performance of the novel biosensors in general.

In contemporary research, the use of cross-linkers has widely opened its way to commercial products. Many analytical kits offer a further step of treatment with one of

the cross-linking agents. This recommended extra step clearly reveals the importance of covalent immobilization in detection procedures. EDC belongs to the family of carbodiimides (dehydrating chemicals) and is one of the well-known crosslinking agents that, in association with NHS, can be used for modification of surfaces by conversion of –COOH groups to NHS-ester compounds. Intermediate ester groups are highly reactive towards $–NH_2$ groups of peptide sequences (originating from Lysine within Dengue Ab molecules in present case), thus producing covalent immobilization of biomolecule to the surface. This method of covalent binding of proteins has been widely used in both biomaterials applications and surface-designed biosensors (Saralidze et al., 2007). Possibly the optimal surface concentration of –COOH groups in our experiments exists on PMMA-co-MAA (9:1) coating. As a justification for such observation, PMMA-co-MAA (5:5) with the highest concentration of –COOH surface groups, demonstrated significantly lower intensity of detection signal as high concentration of surface functional groups is not always desirable (Fig 5.7). As a consequence of an overly functionalized surface, proteins can deactivate due to a steric repulsion (Hosseini et al., 2014; Hosseini et al., 2014c; Hosseini et al., 2014d, Hosseini et al., 2015). However, the covalent immobilization of Dengue Ab molecules on minimal concentration of –COOH surface groups of PMMA-co-MAA (9:1) has resulted in higher detection signals almost for all the aging categories (Fig 5.7).

Considering our observation during the assay, along with the statistics in Fig 5.7, we can conclude that the physical immobilization presents more reliable and reproducible method in comparison to covalent immobilization. Chemical binding of the proteins via EDC/NHS chemistry (covalent attachment) is not always reproducible and is often difficult to control (Sam et al., 2009). Previous reports on precise analysis of EDC/NHS treated surfaces revealed that, in particular cases, such treatment could lead to formation of anhydride groups (instead of desirable NHS-ester groups), which are

totally unreactive toward amines or proteins (Sam et al., 2009; Wang et al., 2011). Other previous publications also pointed to the precautions and possible drawbacks of such approach, as in some cases, EDC/NHS can cause an early cross-linking inside the individual protein molecules (Situma et al., 2005). Such an undesirable effect results in the loss of protein activity, which consequently causes poor detection signal and significant loss of sensitivity (Situma et al., 2005). Nevertheless, use of EDC/NHS treatment can still be useful in special cases.

It is important to note that developed biochips in this study have significantly smaller surface areas available for biomolecule binding in comparison to the surrounding walls and bottom of 96 well-plates used as clinical standard kits (Fig 5.1). Despite the smaller surface reaction area, our results show significantly higher detection signal recorded from polymer-coated silicon chips in all cases. Possibly the best way to enhance the detection signal in ELISA experiments is the usage of polyacrylate microspheres, which are fabricated from the same family of copolymers (Hosseini et al., 2014d). Apart from their tuneable surface chemistry the application of microspheres significantly increases specific surface area available for protein interaction. Since the microspheres can be produced by free radical polymerization, the same ageing stability and shelf-life can be expected. Presented biochips as well as polymethacrylate microspheres can further be integrated inside the microfluidic platforms, creating many new possibilities for fabrication of portable diagnostic devices. We have produced the polymeric materials that can be potentially used not only as highly sensitive polymeric coatings in analytical applications but also for fabrication of diagnostic tools such as analytical kits and microfluidic devices. Results presented in this chapter reveal the fundamental aspects that define an optimal polymer substrate with controlled characteristics, stability and great potentials for biosensors applications.

5.5 Conclusion

In this chapter, PMMA-co-MAA coated silicon chips in different molar ratios of the monomers (MMA/MAA) have been prepared as polymeric platforms for biomolecular recognition. Detection of DV was performed on two different types of investigated surfaces: (1) adsorptive physical attachment of Dengue Ab molecules to the surface; and (2) covalent attachment of Ab molecules to the surface via carbodiimide chemistry. In either case, higher detection intensity was achieved. Our results were compared with conventional ELISA test and the coatings demonstrated an increase of approximately 2 times in signal intensity by either method: physical and covalent immobilization.

Although chemical attachment of the Ab molecules is as efficient as physical immobilization, the method involves a complex protocol with the risk of compromised reproducibility. By presence of surface –COOH groups, relying simply on physical adsorption of the Ab to the PMMA-co-MAA surfaces is not only easier and less time consuming, but also offers a higher chance of reproducibility and cost-effectiveness. More importantly, our results have introduced a straight-forward method with a great potential for producing a new generation of ELISA well-plates or fabrication of microfluidic devices with reasonable degree of control over surface properties. As one of the major objectives of this research, the aging effect of the samples with different lifetimes has been examined and results have confirmed that no considerable changes occurred on the polymer surfaces over the period of up to 9 months.

The results reported in this chapter open possibilities for further actions to improve the signal intensity; this can be achieved by enhancing the contact surface of PMMA-co-MAA in each well. The most straightforward method would be to use polymer microspheres with exposed carboxyl groups at their surface.

They offer significantly higher specific surface for Ab-plasma contact and anticipated increase in detection signal. Microspheres sink in each well, so that easy removal of supernatants by pipetting is technically possible. Further work into this direction is currently ongoing in our laboratories. The major challenge will be to convert these results into advanced systems for continuous detection of DV, such as microfluidic devices. This fundamental approach opens many possibilities and strategies in production of more sensitive diagnostic devices for detection of all kinds of viruses not just limited to DV.

CHAPTER 6

Synthesis and processing of ELISA polymer substitute: the influence of surface chemistry and morphology on detection sensitivity

6.1 Introduction

Despite the known drawbacks of Enzyme-linked immunosorbent assay (ELISA), one of the deficiencies that have relatively been ignored is the performance of ELISA substrate itself. Polystyrene (PS) and PMMA, as the cost effective materials of choice for mass production of ELISA well-plates, has shown obvious lacks of suitable physical and chemical properties for protein attachment. The general concept of this work was to develop a potential substrate that can be suggested as a material of choice for production of a new generation of ELISA analytical kits. Spin-coated thin films of polymethylmethacrylate-co-methacrylic acid (PMMA-co-MAA) on silicon surfaces were designed and processed for detection of Dengue virus (DV) as biochips. Coated surfaces of different molar ratios have been investigated as carboxyl-functionalized layers for obtaining platform for biomolecule immobilization with high level of protein activity. To improve the sensitivity of detection, we have used amine functional "spacers", hexamethylenediamine (HMDA) and polyethyleneimine (PEI), which were covalently bonded to the surfaces of PMMA-co-MAA coatings. Results demonstrate that the variation of surface concentration of carboxyl groups of PMMA-co-MAA can be used to control the amine surface concentration after carbodiimide coupling with HMDA and PEI spacers. The presence of amine spacers increases hydrophilicity of the coatings and significantly impacts the polymer surface morphology. In particular, protein immobilization via amine-bearing spacers has been achieved in two effective steps: (1) carbodiimide bonding between amine spacer molecules and PMMA-co-MAA polymer coatings; and (2) covalent immobilization of antibody via glutaraldehyde reaction with amine groups from amine-

treated surfaces. The application of PEI spacer in comparison to HMDA has shown much higher intensity of detection signal in ELISA experiment, indicating better immobilization efficiency and preservation of antibody activity upon attachment to the polymer surface.

6.2 Literature review

Perhaps one of the most commonly applied diagnostic tools for virus detection is enzyme-linked immunosorbent assay (ELISA). In addition, ELISA is also utilized in variety of applications such as determination of food allergens, concentrations of antibody in blood serum, concentrations of specific types of drugs and detection of cancer biomarkers (Kirsch et al., 2013; Lin et al., 2010). Apart from all of the shortages of ELISA assay such as time consuming, tedious procedure and inconsistency of the results (Xu et al., 2006), in particular, the detection limits in this analytical method is a major issue when early diagnostics and successful treatment of patients are aimed (Lai et al., 2004). In some cases, the early diagnosis of infection is considered as the most important factor for successful treatment of infected patients (Ying Liu et al., 2012). Many diagnostic devices depend on careful surface design of the functional polymeric platforms (Audouin et al., 2012; Bucatariu et al., 2013; Yonamine et al., 2012). The modern biosensors are based on heterogeneous processes such as immobilization of antibodies/antigens on the detector surface and measurement of the generated signal through highly specific binding between the surface and the aqueous analyte (Jung et al., 2008). For that reason, the production of functionalized surfaces with high degree of control over surface properties (chemistry and morphology) presents the most important aspect in the development of efficient biosensors (Hosseini et al., 2014; Mitchell, 2011).

One of the materials of choice in biosensors research is polymethylmetacrylate (PMMA). A great number of research papers report the generation of carboxyl (–COOH) functional groups on PMMA plastic by surface modification techniques such as wet chemical (Bai et al., 2006; Brown et al., 2006; Varma et al., 2003), UV radiation (Situma et al., 2005) and

plasma treatments (Coad et al., 2013; Li et al., 2005a; Tennico et al., 2010; Vesel et al., 2012; Vesel & Mozetic, 2012). Those methods might be effective in reported cases but there are still some major concerns about such treatments. Apart from plasma polymerization technique that offers great deal of control over surface properties, wet chemistry treatments as well as the exposure of PMMA to UV light make insignificant changes on the surface. In such cases, "functionalized surfaces" last for a very brief periods of time in which the polymer surface energy goes back to a minimum level by means of "surface re-organization" (Hosseiniet al., 2014a; Hosseiniet al., 2014b). Instead of applying relatively uncertain surface modification methods for generation of desirable functional groups on PMMA plastics, we have processed the coatings with synthesized copolymer by using methylmethacrylate (MMA) and methacrylic acid (MAA) monomers in varying concentrations. With this approach there should be no doubt about the stable existence of inherent –COOH groups at the outermost surface layers of polymethylmethacrylate-co-methacrylic acid (PMMA-co-MAA) copolymers (Hosseini et al., 2014a).

In this chapter we present the PMMA-co-MAA coated silicon chips with controllable content of –COOH groups for Dengue virus (DV) detection by means of covalent immobilization. Dengue virus belongs to the family of *Flaviviridae,* which is a Mosquito-borne viral infection. According to the estimation of the World Health Organization (WHO), the number of the DV infections exceeds to 50 million annually (Alcon et al., 2002; Stevens et al., 2009). Self-limiting febrile disease called Dengue fever (DF) can be associated with life-threatening manifestations such as Dengue hemorrhagic fever (DHF) and Dengue shock syndrome (DSS), which are often ascribed to 1-5% and 10-30% of the recorded cases, resulted in death (Xu et al., 2006). We have used the functional polymeric surfaces to introduce amine spacers of two sizes (Figure 6.1).

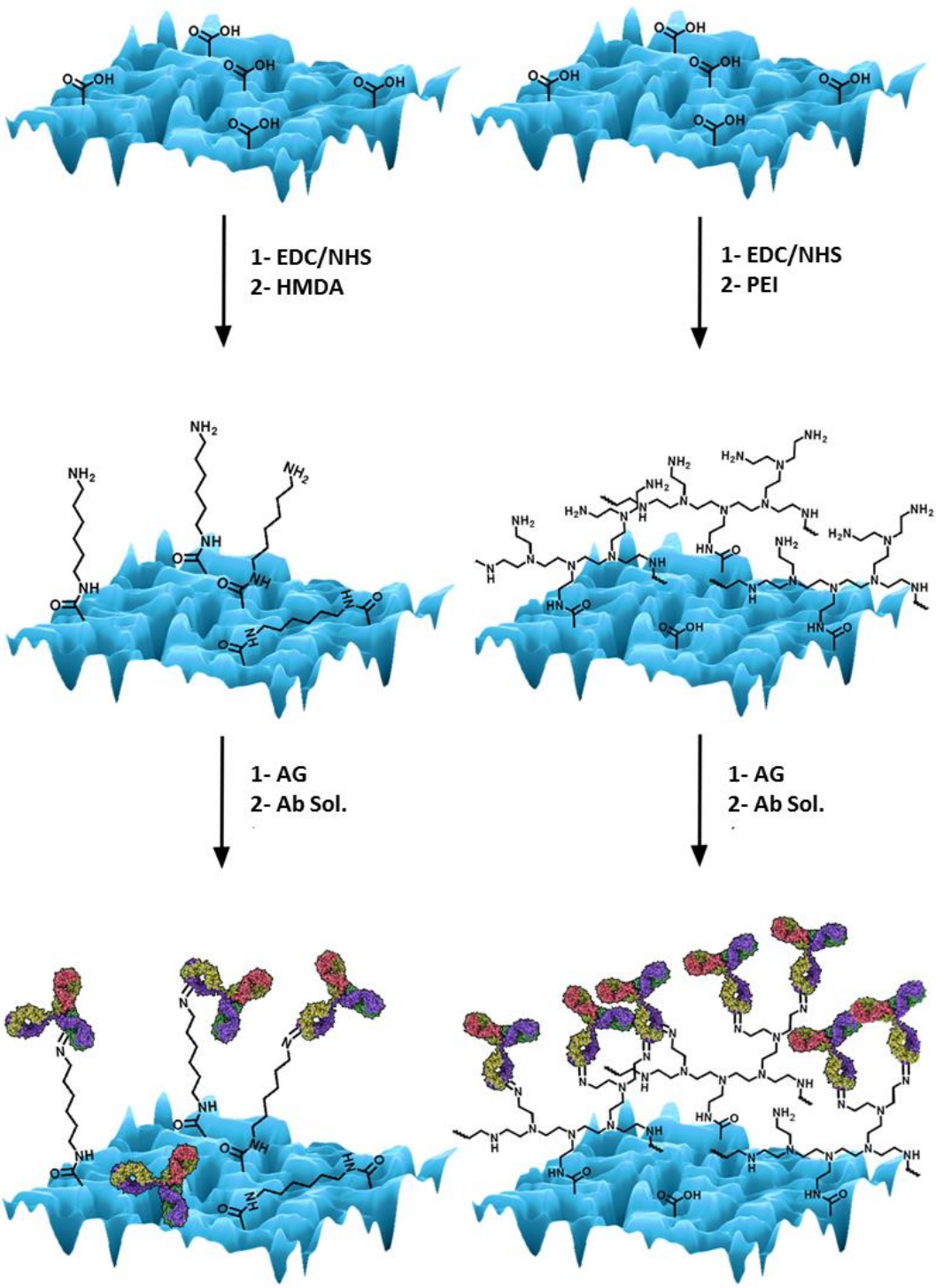


Figure 6.1: Dengue antibody (Ab) binding on PMMA-co-MAA coated surfaces, treated with hexamethylenediamine (HMDA) and polyethyleneimine (PEI) amine spacers: (left) surface amination with HMDA in two steps (step 1-EDC/NHS and formation of NHS ester; step 2-reaction between HMDA amine groups and PMMA-co-MAA surface carboxyl groups; Ab immobilized with glutaraldehyde (GA) reaction (step 1) and subsequent incubation of coated chips in Ab solution (step 2); (right) the same procedure (described for HMDA) has been applied for PEI amine spacer and the subsequent Ab immobilization through GA coupling.

Namely, hexamethylenediamine (HMDA-short aliphatic chain spacer) and polyethyleneimine (PEI-branched-poly-spacer) were covalently bonded with PMMA-co-MAA surfaces by means of carbodiimide chemistry. Aminated coatings were interacted with antibody (Ab) molecules in order to study the influence of amine surface spacers on Ab activity. In case of aminated surfaces, free amine ($–NH_2$) groups were treated with glutaraldehyde (GA) solution for subsequent covalent attachment of Ab molecules. DV detection via spaced-out binding of Ab molecules is described in regards to results, obtained from colorimetric ELISA detection.

6.3 Experimental procedure

6.3.1 Chemicals and reagents

Methyl methacrylate (MMA), methacrylic acid (MAA), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), N-hydroxysuccinimide (NHS), polyethylenimine (PEI) (Appendix Table 7), hexamethylenediamine (HMDA) (Appendix Table 8), glutaraldehyde (GA), bovine serum albumin (BSA), monosodium phosphate (NaH_2PO_4), Tween 20 and disodium hydrogen phosphate (Na_2HPO_4) were purchased from Sigma, US. MMA monomer was purified by distillation method prior to the free radical polymerization reaction (Appendix Figure 1). Other materials have been used as received. Tetrahydrofuran (THF), which has been used as solvent in polymer synthesis and processing procedures and phosphate buffer saline (PBS) were purchased from Thermo Fisher Scientific, US. The free radical initiator azobisisobutyronitrile (AIBN) was purchased from Friedemann Schmidt Chemical, Germany. Diced silicon OFET substrates (2cm x 2cm) were purchased from Ossila, UK.

6.3.2 PMMA-co-MAA synthesis and processing

Different compositions of the PMMA-co-MAA copolymers have been synthesized in free radical polymerization reaction. Synthesized polymers have been used for fabrication of

spin-coated silicon wafers by previously reported method (Appendix Figures 2 and 3). (Hosseini et al., 2014a). In brief, free radical polymerization of MMA and MAA monomers has been completed in following molar ratios: pure PMMA, PMMA-co-MAA-(9:1), PMMA-co-MAA-(7:3) and PMMA-co-MAA-(5:5). Apart from pure PMMA, the numbers in brackets indicate initial MMA/MAA molar ratios. Polymer coatings were prepared on silicon wafers (size 2 cm × 2 cm) by spin-coating procedure (Appendix Figure 4). Cleaned silicon wafers (substrates) have been coated by using spin-coater (Laurell, model WS-650MZ-23NPP) with spinning time of 55 seconds at 3000 rpm (5% polymer solutions in THF) (Hosseini et al., 2014a).

6.3.3 Surface modification-amination with HMDA and PEI

The Surface modification of spin-coated silicon chips (PMMA-co-MAA) has been achieved by following steps: (1) chips were incubated in EDC/NHS solution (0.155 g of EDC and 0.115 g of NHS in 200 ml of PBS) for 1 hour; (2) samples were taken out and thoroughly washed with PBS buffer; (3) samples have been incubated for 1 hour in amine spacer solutions (0.1% PEI in PBS or 0.1% HMDA in PBS) followed by washing in PBS; (4) aminated samples were treated with GA solution (4% in distilled water; 30 minutes) and subsequently incubated in primary Ab solution as first step of the ELISA assay. The amination treatment was performed in room temperature. Samples were thoroughly washed prior to sandwich ELISA assay (described in Appendix section S.14). It should be noted that deposition of the amine layers on PMMA coatings has occurred through physical adsorption since the –COOH groups do not exist on the surface of PMMA-coated chips.

6.3.4 Scanning electron microscopy (SEM)

The morphology of the coated surfaces of chips was analyzed by SEM equipped with a field emission gun (FESEM, JEOL, JSM7600F), which was operated at an accelerating

voltage of 0.5 kV. The polymer coated samples were placed on a double-sided conductive tape and the images have been recorded in secondary electron mode. The samples have been incubated in PBS buffer for 1 hour prior to cross-section imaging and the SEM images have been obtained after incubation.

6.3.5 Atomic force microscopy (AFM)

AFM (Ambios, Q scope) has been used to record the surface images of coated chips before and after amination treatments. The morphology of the surfaces has been analyzed in contact mode. Mean roughness (Ra, nm) and root mean square roughness (Rq, nm) were measured for all polymer surfaces.

6.3.6 Water-in-air contact angle measurement

The water-in-air contact angle of the surface of chips was measured in room temperature applying sessile drop method. Dataphysics Contact Angle System (OCA) instrument has been used for this experiment. The contact angle was measured in first minute after a drop of water (0.1 µL) was placed on the polymer coated substrate. Reported values are the average of different measurement of five separate drops, on the center and four corners of the surfaces. Calculated standard deviation is presented with the results (n=6).

6.3.7 X-ray photoelectron spectroscopy (XPS)

The XPS measurements have been carried out in a Quantera SXMtm from Ulvac-PHI (Q1). The measurements have been performed using monochromatic AlKα-radiation and a take-off angle θ of 45°; at this angle the information depth is approximately 7 nm. A spot size of 300×500 µm was applied for the analysis. By means of wide-scan measurements the elements present at the surface have been identified; accurate narrow-scans have been measured for precise quantification and identification of chemical states. Standard sensitivity factors were used to convert peak areas to atomic concentrations. As a result, it

is possible that the concentrations deviate from reality in the absolute sense (generally not more than 20% relative).

6.3.8 Immobilization of Dengue antibody molecules in sandwich ELISA

The sandwich ELISA using colorimetric method was performed in order to assess the potential of polymer coated silicon substrates as platforms for DV detection (detailed description of the ELISA assay can be found in chapter 5 or Appendix section). In brief, goat IgG anti Dengue virus 2 (D-15): SC-325014 and mouse IgG anti Dengue virus ICL2: SC-65725 (Santa Cruz) were chosen as capture and primary antibodies, respectively and anti-mouse IgG (Fc specific, A-11001, Alexa Flour) was used as secondary antibody. Apart from conventional ELISA, which has been performed as control, two experiments have been conducted: (1) physical adsorption of Ab on PMMA-co-MAA coatings (untreated surfaces) and (2) covalent immobilization of Ab to the surface $–NH_2$ groups of aminated coatings (amine treated surfaces). The coated silicon chips were carefully cut to fit into the wells (4mm x 4mm) of 96-well plates (SPL, life science, Korea). The total of 12 positive and 8 negative replicates of each composition have been examined in sandwich ELISA.

6.4 Results and discussion

6.4.1 Surface morphology

In our previous work we presented PMMA-co-MAA coatings produced from the synthesized polymer with variation of initial molar ratio of MMA/MAA. The film thickness recorded was in the range of 10-20 µm, which is consistent with current experiment (Fig. 6.2e insert) (Hosseini et al., 2014a). The free surface –COOH groups were used for covalent reaction with amine spacers (HMDA and PEI) through carbodiimide chemistry. Figure 6.2 presents the SEM images of aminated surfaces of the PMMA-co-MAA coatings. The frontal view of the coated chips have shown uniform

morphology for HMDA aminated PMMA, while PMMA-co-MAA with molar ratio of 9:1 reveals more rough surface after amination with HMDA (Fig. 6.2a and b). Coating delamination can be observed on the outmost layer of both PMMA-co-MAA-(7:3)-HMDA and PMMA-co-MAA-(5:5)-HMDA (Fig. 6.2c and d). Furthermore, in detailed imaging of coatings cross-sections for the mentioned surfaces, multilamination of the coated layers can be seen as well (Fig. 6.2e-h). The cross section images show the comparison between the coatings thickness of untreated and aminated coatings with HMDA (Fig. 6.2e-h and inserts).

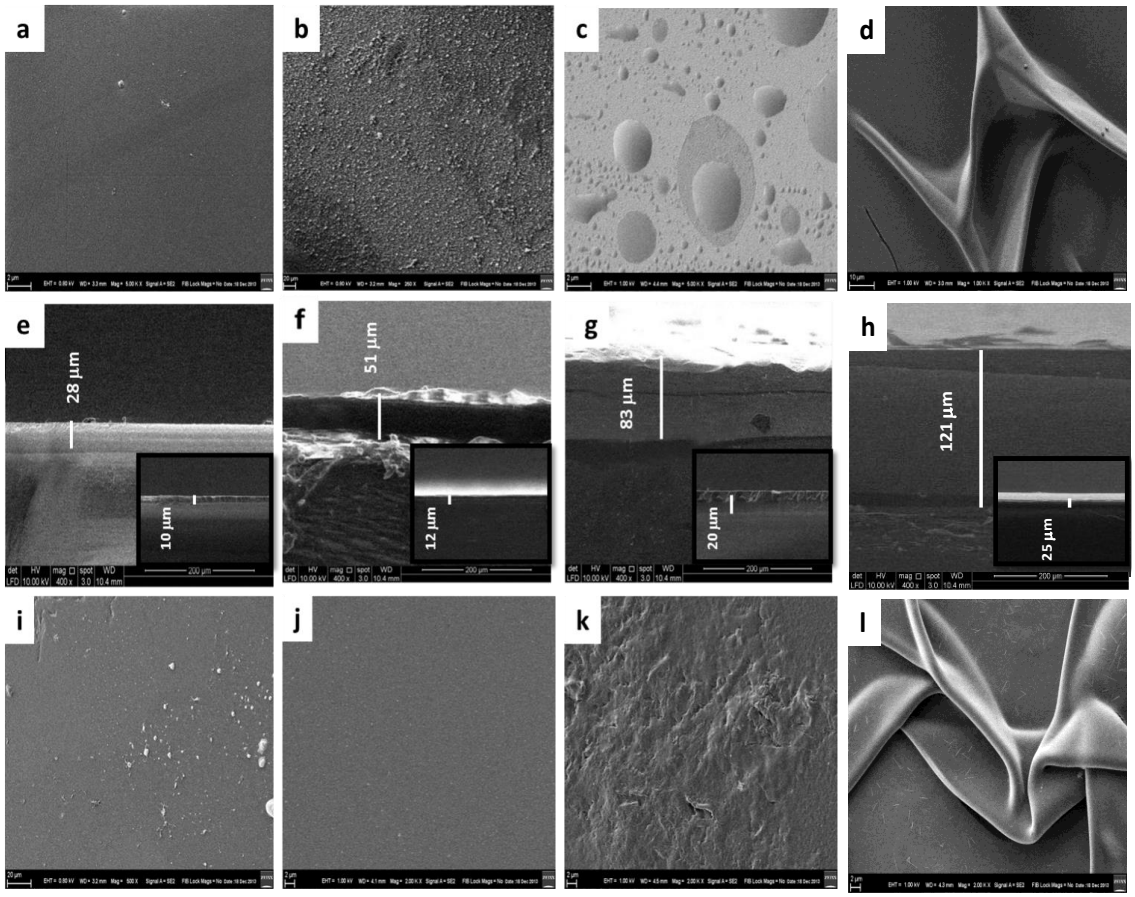


Figure 6.2: SEM images of polymer coated chips; top row: frontal view of HMDA treated coatings (a) PMMA; (b) PMMA-co-MAA (9:1); (c) PMMA-co-MAA (7:3); and (d) PMMA-co-MAA (5:5), (middle and bottom rows follow the same sequence of PMMA-co-MAA); middle row: cross section images of HMDA treated coatings (e-h) and untreated coatings (e-h inserts) after incubation in PBS for 1h at 25°C; bottom row: the frontal view of PEI treated PMMA-co-MAA coatings (i-l).

In order to assess the influence of coating thickness and morphology of the coated surfaces on Ab immobilization, all the samples in Fig. 6.2 (e-h, including inserts) have been incubated for 1 hour in PBS and washed thoroughly with distilled water prior to SEM cross-section imaging. There is a significant increase in coating thickness due to the existence of hydrophilic HMDA layer, which is also in line with our previous findings. From visual observation, PMMA-co-MAA (5:5) coating turns into a swollen gel-like surface in all the experiments due to the high concentration of hydrophilic MAA segments (Hosseini et al., 2014a; Saunders et al., 1997). Figure 6.2 (e-h) shows an increase from the original thickness of 28-29 µm (PMMA-HMDA; Fig. 6.2, e) to 118-121 µm for PMMA-co-MAA-(5:5)-HMDA (Fig. 6.2h). Similar trend has been observed for PEI aminated PMMA-co-MAA coatings (Appendix Figure 5). Unmodified coatings also showed increased swelling but in significantly lower degree (inserts, Fig. 6.2e-h). The frontal view of PEI aminated coatings shows the smooth and uniform surface morphology for PMMA-PEI and PMMA-co-MAA-(9:1)-PEI (Fig. 6.2i and j) while the other two coatings are composed of irregular surface features (Fig. 6.2k and l). Coating delamination similar to Fig. 6.2d also appears on PMMA-co-MAA-(5:5)-PEI sample (Fig. 6.2d and l).

Further surface analysis with AFM presented in Fig. 6.3 revealed consistent morphologies for both pure and HMDA-treated samples. Obvious surface alterations can be observed on PEI-treated coatings as a consequence of the relatively thick layer of PEI macromolecules (Fig. 6.3j-l). Table 6.1 provides the information about mean roughness and root mean square roughness on the treated and untreated surfaces detected by AFM. Apart from pure PMMA coatings, HMDA and PEI treated surfaces of other compositions have shown significantly higher surface roughness in comparison to untreated samples (Table 6.1). For reminder, PEI macromolecules have been immobilized on polymer coatings through covalent coupling in corbodiimide reaction between surface –COOH groups of PMMA-co-MAA and PEI amine (-NH_2) groups (Figure 6.1).

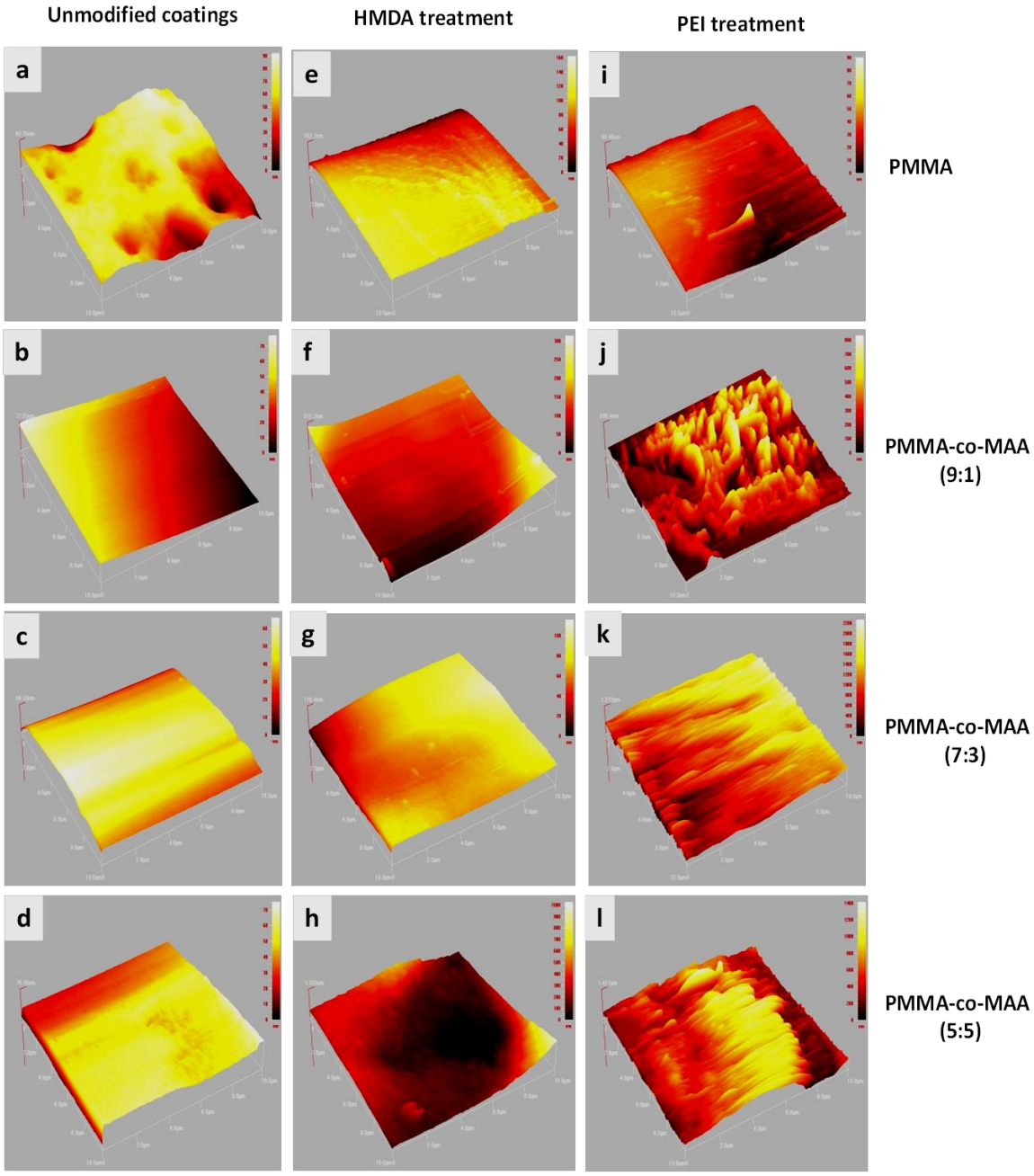


Figure 6.3: AFM topography of polymer coated chips: (a-d) as-prepared (unmodified) PMMA-co-MAA coatings; HMDA (e-h) and PEI (i-l) treated coatings.

PEI treated surface of the PMMA-co-MAA-(9:1) has significantly different surface features among all of the treated and untreated surfaces (Fig. 6.3j). The increase in specific surface available for interaction with biomolecules is expected to have a significant influence on activity and concentration of surface-immobilized proteins.

Table 6.1: Surface morphology data determined by AFM.

Composition	Mean roughness (Ra, nm)	Root mean square roughness (Rq, nm)
PMMA	11.3	13.2
PMMA-co-MAA 9:1	12.3	14.2
PMMA-co-MAA 7:3	2.69	3.11
PMMA-co-MAA 5:5	3.82	4.86
HMDA trearted coatings		
PMMA	10.7	13
PMMA-co-MAA 9:1	24.6	31
PMMA-co-MAA 7:3	43.2	51.7
PMMA-co-MAA 5:5	90.2	112
PEI treated coatings		
PMMA	7.8	8.77
PMMA-co-MAA 9:1	91.2	111
PMMA-co-MAA 7:3	311	361
PMMA-co-MAA 5:5	203	235

6.4.2 Surface chemistry

Water-in-air contact angle (WCA) was measured for HMDA and PEI treated surfaces of polymer coatings in comparison to the previously reported results on pure PMMA-co-MAA (Hosseini et al., 2014a). More drastic decrease in WCA can be observed (Fig. 6.4a) for aminated samples with respect to the increase in surface concentration of –COOH groups, originating from controlled chemical structures of PMMA-co-MAA.

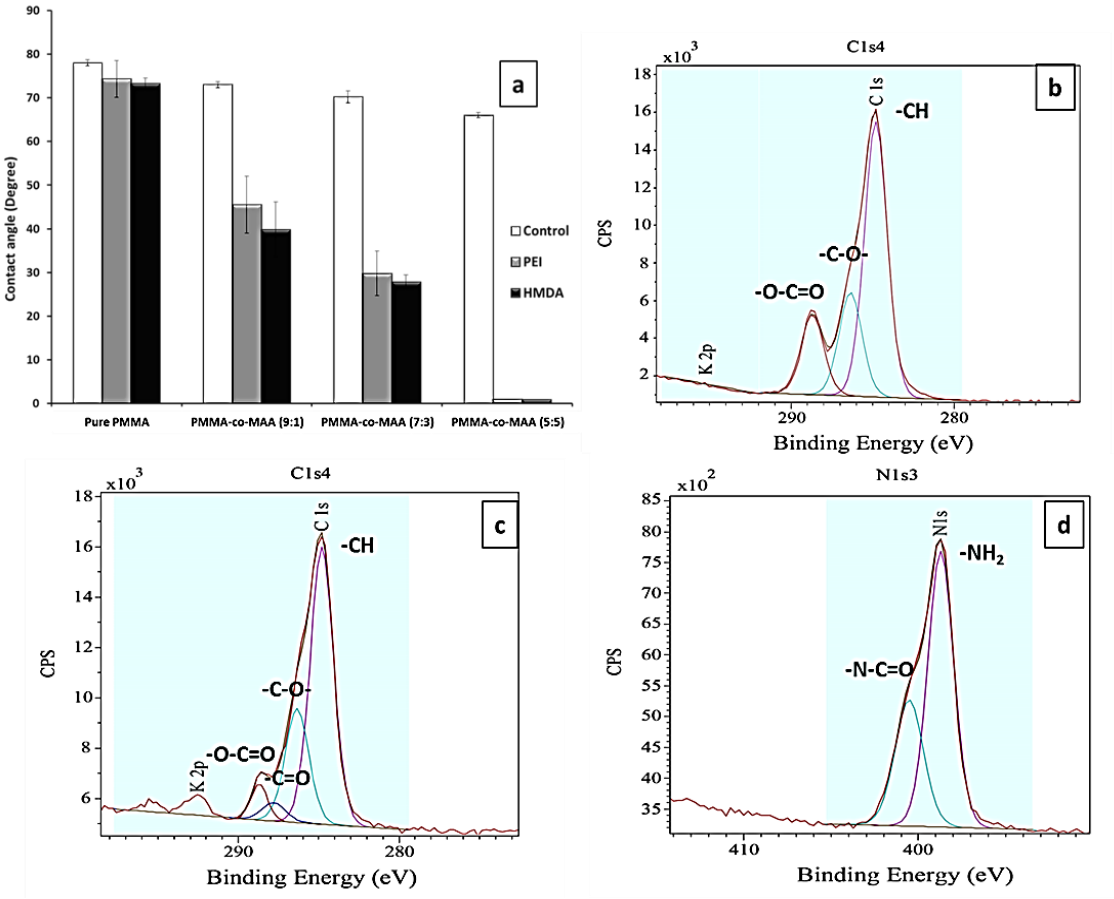


Figure 6.4: Surface analysis of PMMA-co-MAA coatings and the influences of HMDA and PEI amine spacers on hydrophilicity and surface chemistry: (a) water-in-air contact angle ("control" represents as-prepared polyacrylate coatings without treatment); (b) XPS C1s representative peak for PMMA-co-MAA coatings without HMDA/PEI treatments; (c) XPS C1s peak of representative PMMA-co-MAA-HMDA showing the additional peak at 287.8 eV; (d) N1s peak of PMMA-co-MAA-PEI showing two peaks assigned to amide (–N–C=O) and amine ($–NH_2$) groups at 400.8eV and 399.4eV, respectively.

Since the PMMA structure (and the surface respectively) does not present –COOH functionalities available for EDC/NHS reaction with amines, only a relatively small drop in WCA value (~5˚) was observed for PMMA in comparison to the other surfaces (Fig. 6.4a). In particular, PMMA-co-MAA-(5:5) with the highest concentration of –COOH groups, treated with both HMDA and PEI, presents an extremely high hydrophilicity with the WCA of only several degrees, which was beyond the measurement for the instrument.

The chemical composition of PMMA-co-MAA surfaces was analyzed with XPS and the shape and position of the C1s-peaks (Fig. 6.3b) is typical for PMMA. New peaks, assigned

to –C=O groups were identified on all aminated surfaces and the representative C1s peak is shown in Fig. 6.4c. The expected N1s peaks were also detected after PEI amination (Fig. 6.4d). Both C1s and N1s peaks were curve-fitted and apparent concentrations at the outer surface of the samples are presented in Table 6.2. Peak positions in eV and the chemical assignments are indicated in the top row (Table 6.2. "-"denotes that the concentration is less than the detection limit: < 0.1 at %).

Table 6.2: Surface concentrations of (in at %) of the investigated samples by XPS: pure PMMA-co-MAA, HMDA-treated and PEI-treated surfaces (small concentrations of Na, K, Cl and some organic Si were neglected in presented data).

Peak	**C 1s**				**N 1s**		**O 1s**
Binding energy (eV)	284.8	286.4	287.8	288.7	400.8	399.4	530
Peak assignment	**-CH**	**-CO**	**-C=O**	**O-C=O**	**-N-C=O**	**$-NH_2$**	
PMMA-HMDA	42.5	17	-	11.5	-	0.8	25
Comp.(9:1)-HMDA	42.5	15.5	1.7	10	-	1.7	25.5
Comp.(7:3)-HMDA	42.5	17	1.8	4.5	-	4.6	24.5
Comp.(5:5)-HMDA	37	14	3.1	2.6	-	3.1	32
PMMA-PEI	49.5	12	2.6	0.5	1	8.2	19.5
Comp.(9:1)-PEI	44	14.5	3	5	1.7	5.1	21
Comp.(7:3)-PEI	47.5	11.5	3.8	3.5	1.9	5.1	19
Comp.(5:5)-PEI	54.5	7.5	6.2	1	3.4	8.4	15.5

In case of HMDA treated coatings, the amount of nitrogen increases proportionally with an increase of –COOH surface concentration (compositions (9:1) and (7:3); Table 6.2). The peaks assigned as " –C=O" groups in Table 6.2 (detected at 287,8 eV; Fig. 6.4c) could be interpreted as newly-created amide bonds after carbodiimide coupling (Kilian et al., 2007). From our results there are no such groups detected for PMMA, but only for PMMA-co-MAA with evident surface –COOH groups available for conversion to amides in EDC/NHS binding. The PMMA-co-MAA substrates are covered with thin film of several nanometers with aligned concentrations of $–NH_2$ surface groups from HMDA (Table 6.2).

For the sample with highest concentration of –COOH groups, PMMA-co-MAA-(5:5), there is a decrease in $–NH_2$ surface concentration after amination. This particular sample also shows an increase of –C=O concentration (287,8 eV). Due to the high concentration of –COOH groups, both $–NH_2$ of HMDA might have reacted with abundant –COOH functionalities present on such surfaces (Figure 6.1, left column). Increased concentration of HMDA in amination treatment further enhances undesirable binding with $–NH_2$ groups of diamine molecule. It is important to note that no amide peak has been detected in curve-fitted N1s peak of HMDA treated surfaces so such interpretation could remain in question. In any case, the presence of hydrophilic amine coating has a significant effect on both WCA (Fig. 6.4a) and the apparent swelling after incubation in PBS (Fig. 6.2e-h).

For PEI-treated surfaces, there is an obvious appearance of new N1s peak detected at 400.8 eV (Fig. 6.4d) that could be assigned to both imide (O=C–N–C=O) and amide (–N–C=O) groups (Kilian et al., 2007; Xiao et al., 1997). Representative detected N1s peak for HMDA-treated surface can be found in Appendix Figure.6. Imide groups are the product of EDC/NHS reaction with –COOH surface functionalities, called the "NHS ester"groups. The intermediate NHS esters have been reported as semi-stable and highly-reactive towards $–NH_2$ groups and by the time of the XPS experiment, all NHS esters were most likely hydrolyzed. It is important to note that EDC/NHS reaction with surface –COOH groups could also lead to formation of N-acylurea and subsequent detection of imide peaks (Sam et al., 2009). In this case, the atomic percentage of $–NH_2$ increases proportionally with C1s (-C=O) and N1s (-N-C=O) peaks at 287.8 eV and 400.8 eV respectively (Table 6.2), which indirectly proves the efficiency of the amination treatment where the concentration of amide bonds is proportional to the concentration of free $–NH_2$ groups at the surface (Ghasemi et al., 2011; Kilian et al., 2007).

The shape of the C1s-peak for aminated surfaces is quite different from the shape of the C1s-peak of PMMA (Fig. 6.4b and c), which also demonstrates that an organic layer of PEI is present with a thickness larger than 7 nm. This highly hydrophilic polymer layer (Fig. 6.4a) can have a significant influence on surface binding of biomolecules used in biosensor devices. Although hydrophobic nature of the bioreceptor surface might have a considerable influence of protein immobilization through hydrophobic interaction, surface hydrophilicity of the developed platforms can also facilitate the immobilization in an indirect manner. For instance, in the present case, the surface hydrophilicity of the samples are the function of available –COOH functional groups. In the other words, higher hydrophilicity of the samples is equivalent to increased concentration of surface –COOH groups derived from MAA segments. Available –COOH functionalities, in turn, facilitate protein immobilization by another means, hydrogen bonding. Presence of such surface functionalities imposes the platform to react with the targeted analyte via hydrogen bonding. This force has found to be significantly effective in immobilization procedure than hydrophobic interaction (Yoon et al., 1997). As a result, in the present case, hydrophilicity of the surface indirectly, encourages the surface to bind to the biomolecules by a substantial mean.

6.4.3 Dengue virus detection in colorimetric sandwich ELISA

6.4.3.1 Antibody attachment through spacer-glutaraldehyde reaction

Generally, spacers are the molecules with available functional groups that can be used for coupling of the proteins with the surface. Linear diamine HMDA molecules have been chosen as a representative of small spacers. In contrast, branched PEI was used as a large molecule for surface treatment. The aminated surfaces have been first exposed to the glutaraldehyde and subsequently incubated in Ab solution (Figure 6.1) (Bai et al., 2006; Gunda., 2013).

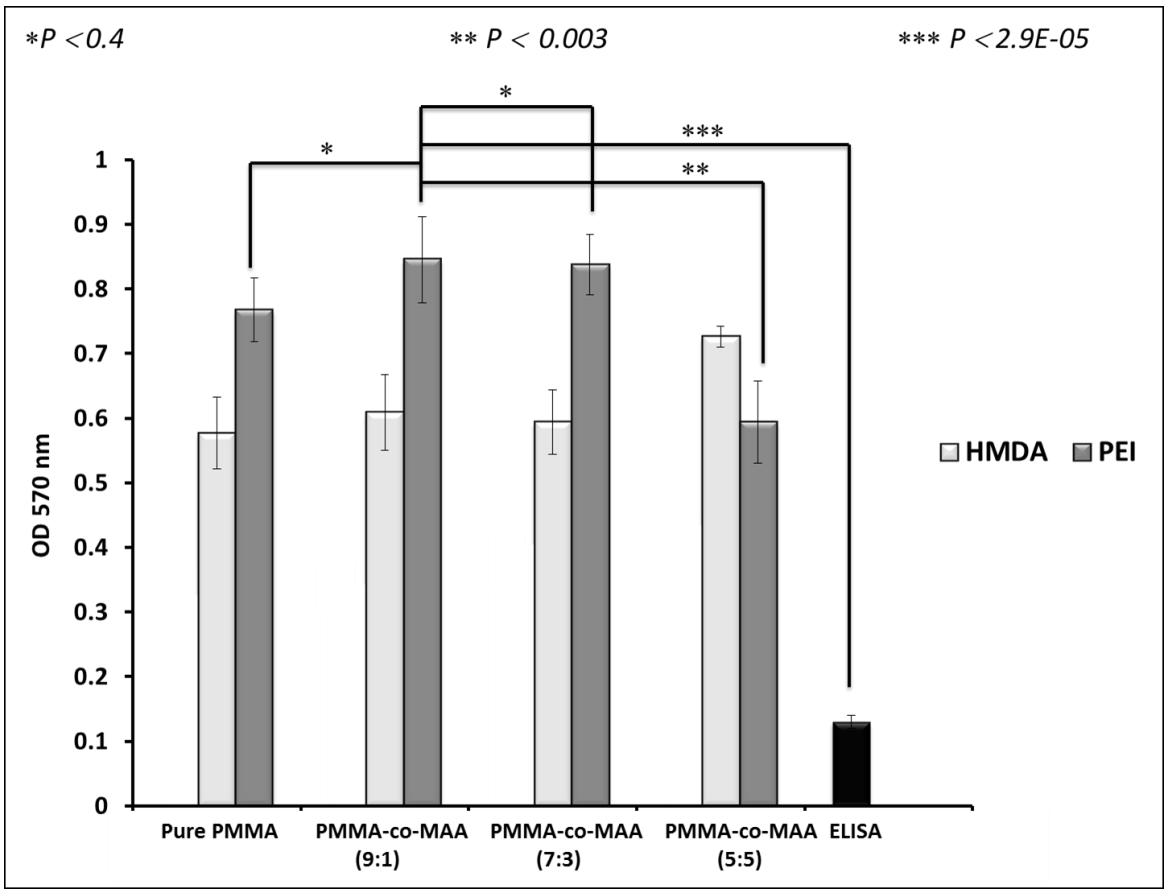


Figure 6.5: Colorimetric ELISA detection signal originating from specific coupling between primary and secondary antibodies from untreated (physical attachment) and amine treated (HMDA and PEI) surfaces. Conventional ELISA detection of DV is shown in black.

From Fig. 6.5, it can be observed that the detection signal substantially increased by PEI treatment comparing other spacer. The larger molecule of the PEI greatly affects the binding efficiency as it provides the sufficient $–NH_2$ groups for further interaction with Ab (Bai et al., 2006; Bucatariu et al., 2013; Coad et al., 2013; Gunda et al., 2014).

HMDA treated coatings have shown relatively lower detection efficiency in comparison to PEI treated surfaces (Fig. 6.5 and Appendix Figure 7). The major difference between two spacers is the size and the concentration of $–NH_2$ groups available for reaction (Figure 6.1). Judging from the size of the molecule, HMDA can only generate $–NH_2$ groups in relatively close proximity to the polymethacrylate surface (Bai et al., 2006).

In contrast, the superior performance of the surfaces that have been treated with PEI (~6-8 times higher signal intensity than conventional ELISA) can only be attributed to the existence of polyamine spacer on the chips. It is interesting to note that a different performance of PEI spacer was occurred only for treated PMMA-co-MAA-(5:5) coatings where PEI treated coating showed lower signal intensity than HMDA (Fig. 6.5). This might be attributed to the fact that the surface of the PEI treated PMMA-co-MAA-(5:5) composition has the "too many" $–NH_2$ functionalities to offer thus causing steric repulsion. Some may ask why the same phenomena did not affect the HMDA aminated surfaces of the same PMMA-co-MAA-(5:5) composition. The answer can be found in the size and the structure of the HMDA spacer molecule, which offers much less available $–NH_2$ functional groups. Such an effect confirms that the highest possible surface concentration of functional groups does not necessary yield an effective bio-functionalization of polymer platforms. ELISA results confirm the hypothesis that only a careful control and optimization of chemical functionalities should provide the qualified surface for further protein immobilization (Hosseini et al., 2014a). For example, too many functional groups on the surface may cause the steric repulsion and subsequent deactivation of biomolecules. Proteins might also become deactivated when the very low concentrations of surface functional groups result in "falling" of proteins on the substrate (Hosseini et al., 2014).

Our experiments on protein immobilization (purified Dengue primary antibody) have shown that the surface of coated silicon chips made of PMMA-co-MAA-(9:1) and PMMA-co-MAA-(7:3) contain the optimum concentration of –COOH functionalities for such a treatment. This is a relatively low surface concentration in comparison to other copolymer composition, PMMA-co-MAA-(5:5). It is reasonable to assume an even surface distribution of –COOH groups on the fabricated coatings since those functionalities are the part of the polymer structures. Lower concentration of surface –COOH groups in comp.(9:1) and comp.(7:3) would possibly allow the limited amide bonding, leaving the

maximum content of unreacted $–NH_2$ groups available for glutaraldehyde-peptide immobilization on PEI treated coatings. Surface morphology of the coated samples should also be taken under consideration. PEI treated coating of PMMA-co-MAA-(9:1) with highest detection signal intensity in ELISA experiment (Fig. 6.5) has shown a stable surface with no sign of delamination in SEM analyses (Fig. 6.2) while it offers different surface topology in comparison to the rest of the examined samples as well (AFM experiment; Fig. 6.3 j). This particular morphology allows more contact of proteins (in aqueous phase) with the solid substrate, which, in turn, increases the surface concentration of immobilized biomolecules and subsequent increase in detection signal. In terms of contemporary analytical challenges, there is no definite explanation for such observation (Hong et al., 2013). The potential biosensors based on the functional polymer surfaces can only be examined through direct verification of protein activity once the immobilization has been achieved (Coad et al., 2013; Kirsch et al., 2013). For that reason the controlled surface properties of such devices is one of the key aspects that would eventually define protein-surface interactions in detail.

6.5 Conclusion

PMMA-co-MAA coated silicon chips in different molar ratios of the monomer have been prepared as polymeric platforms for virus detection. The coatings were modified with amine spacers and sandwich ELISA assay was performed on two different types of investigated surfaces: (1) treated surfaces with PEI amine spacers; (2) treated surfaces with HMDA amine spacers and, in both cases, subsequent covalent immobilization of antibody molecules conducted via glutaraldehyde chemical reaction. The efficiency of antigen/antibody binding was significantly higher for the PEI treated surfaces. In comparison to surfaces treated with short amine spacer (HMDA), coated surfaces treated with PEI resulted in signal intensity that was substantially higher than conventional ELISA. Such a result indicates the preservation of antibody activity upon immobilization,

which can be used as a general approach in development of biosensors. Surface modification of polyacrylate coatings with amine-bearing spacers (HMDA and PEI) also resulted in surfaces with modified platforms with high specific surface area available for interaction with biomolecules. Most importantly, our results demonstrate the simple and cost-effective method for production of new generation of ELISA kits with high degree of control over interfacial processes that are still poorly understood. Our findings have shown the optimal surface binding of biomolecules to the solid substrates at which the protein activity remains unaffected to a significantly large extent. Developed surfaces have shown a great potential in DV diagnostics. This novel fundamental approach will open new possibilities and strategies in production of highly sensitive diagnostic devices for detection of viruses, not limited only to DV.

CHAPTER 7: Conclusion

7.1 Introduction

This chapter summarizes the synthesis and processing of PMMA-co-MAA biochips, which have been fabricated for its application in Dengue virus detection. Presented strategy in this thesis has played a vital role in overcoming the existing problems in diagnostic system. Developed platforms have proven to be qualified for successful immobilization of Dengue antibody and subsequent enhanced detection of Dengue virus.

7.2 Conclusions and major contributions

Methyl methacrylate (MMA) and methacrylic acid (MAA) were used for polymerization of different compositions of copolymer (PMMA-co-MAA) via free radical polymerization reaction. Coated chips in different molar ratios of the monomers have been prepared by spin-coating technique as polymeric platforms for virus detection. In particular, Dengue was chosen as targeted virus. Detection signal was recorded as a result of specific coupling between antigen and antibody that eventually leads to detection of the virus. Sandwich ELISA was performed on 3 different types of investigated surfaces of biochips: (1) untreated polymethacrylate coatings (physical adsorption); (2) treated surfaces with EDC/NHS for covalent binding of biomolecule to the surface of the chips and (3) treated surfaces with amine spacers of different sizes and covalent immobilization of antibody molecules conducted via glutaraldehyde chemical reaction.

Results have proven that Dengue detection via physical immobilization of the protein on the surface of the biochips was as efficient as immobilization through carbodiimide chemistry; both cases resulted in higher intensity of detection signal than conventional ELISA assay. However, the efficiency of antibody binding and subsequent Dengue virus detection was significantly higher for the amine treated surfaces. In comparison to untreated surfaces, or surfaces treated with short amine spacer (HMDA), coated surfaces treated with PEI resulted in substantially higher signal intensity than conventional ELISA.

Such a result indicates the preservation of antibody activity upon immobilization, which can be used as a general approach in development of suitable bioreceptor platforms. Surface modification of polymethacrylate coatings with amine-bearing spacers (HMDA and PEI) also resulted in surfaces with enhanced hydrophilicity and high specific surface area that can be considered as a further reason for superior performance of aminated biochips.

Among all different compositions of the chips, PMMA-co-MAA (9:1) has proven to be the surface of high-performance with the optimum number of surface –COOH groups available for the most efficient protein immobilization regardless of the applied methods. This specific composition has also shown enough surface stability in different types of treatments. Moreover, AFM analysis has given high value of surface roughness for this composition, which, in turn, promotes better biomolecular interaction. Furthermore this particular surface has shown no sign of delamination in detailed analysis with SEM which is important in biomedical application as well. Despite having smaller surface area than wells of the analytical kits, developed biochips enhanced the detection signal up to the considerable level than conventional methods.

As further investigation on the coated surfaces, examining the chips with different periods of shelf life has drawn a vital role in overcoming the major concern in contemporary research regarding aging phenomenon. Developed samples such as immediate coated, 3 months, 6 months and 9 months old were carefully characterized in order to study the effect of aging period on the available surface functionality and their activity. Samples have shown no sign of aging and the strong presence of functional groups, which have been created through free radical polymerization, remained the same. This is of crucial importance for future development of proposed compositions into the new generation of ELISA kits. The developed strategy would offer stable platforms that can serve in clinical practices, overcoming the major concern regarding aging effect.

Most importantly, the results demonstrated the simple and cost-effective method for production of new generation of ELISA kits with high degree of control over interfacial processes. Results have shown the optimal surface binding of biomolecules to the substrates at which the protein activity remains unaffected to a significantly large extent. Although developed surfaces show a great potential in Dengue detection, this novel fundamental approach will make new possibilities and strategies in production of highly sensitive diagnostic devices for detection of all kinds of viruses, not only limited to Dengue diagnostics.

7.3 Limitations of the current study and future directions

Although developed polymeric platforms in this study have proven to be qualified bioreceptor surfaces for biodiagnostic applications, the use of silicon wafer as a substrate of choice for coating is not cost-effective for future commercialization of the platform. Observed delamination signs on the coated surfaces also make the choice of materials limited to the specific compositions. For that reason, synthesis and processing of new types of materials with better consistency and robustness is essential. For instance, combination of the electrospun fibers and synthesized copolymer compositions would offer greater specific surface area for analyte-surface interaction and expectedly enhance the detection signal as well (Hosseini et al., 2015).

One of the promising directions is the production of microspheres made from similar polyacrylate system. Microspheres are known for the vast specific surface area that they offer for biomolecular interaction. They have great potentials for integration into the other diagnostic systems such as microfluidic devices. Taking the advantage of the spherical 3D shapes of the spheres in comparison to the 2D platforms described in this study might lead to the significantly enhanced detection signal and possibly early Dengue detection in the near future.

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LIST OF PUBLICATIONS

Review article

- **Samira Hosseini,** Fatimah Ibrahim, Ivan Djordjevic, and Leo. H. Koole. Recent advances in surface functionalization techniques on polymethacrylate materials for biosensor applications. *Journal: Analyst, DOI: 10.1039/c3an01789c.*

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Recent advances in surface functionalization techniques on polymethacrylate materials for optical biosensor applications

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Biosensor chips for immune-based assay systems have been investigated for their application in early diagnostics. The development of such systems strongly depends on the effective protein immobilization on polymer substrates. In order to achieve this complex heterogeneous interaction the polymer surface must be functionalized with chemical groups that are reactive towards proteins in a way that surface functional groups (such as carboxyl, –COOH; amine, $–NH_2$; and hydroxyl, –OH) chemically or physically anchor the proteins to the polymer platform. Since the proteins are very sensitive towards their environment and can easily lose their activity when brought in close proximity to the solid surface, effective surface functionalization and high level of control over surface chemistry present the most important steps in the fabrication of biosensors. This paper reviews recent developments in surface functionalization and preparation of polymethacrylates for protein immobilization. Due to their versatility and cost effectiveness, this particular group of plastic polymers is widely used both in research and in industry.

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Introduction

Biosensors, "biochip" or "lab-on-chip" devices are important to various fields such as food safety, medical diagnostics, environmental regulation, and many others.[1–11] The major principle behind such devices is that the signal is generated by the recognition of an analyte by a bioactive compound and the

Samira Hosseini obtained her MSc degree in 2013 in polymer chemistry at the Department of Chemistry, Faculty of Science, University of Malaya (UM). She is currently in the process of completing her PhD degree in the field of microfluidic biosensors within the Centre for Innovation in Medical Engineering, Department of Biomedical Engineering, UM. Her research interests mainly involve polymeric materials and surface chemistry in BioMEMS diagnostic applications.

Fatimah Ibrahim obtained her PhD degree in Biomedical Engineering from the University of Malaya (UM) in 2005. She was a senior lecturer at Universiti Teknologi MARA and in 1999 she joined the University of Malaya, and was involved in setting up one of the first Departments of Biomedical Engineering in Malaysia. She did her Biological and Micro-Electromechanical Systems (BioMEMS) research attachment at the University of Irvine, California in 2010. She is currently a Professor in the Department of Biomedical Engineering and Head of Centre for Innovation in Medical Engineering, UM. Her research interests include detection and monitoring of diseases, physiological modeling and measurement, biosensors, BioMEMS and artificial intelligence applications in medicine.

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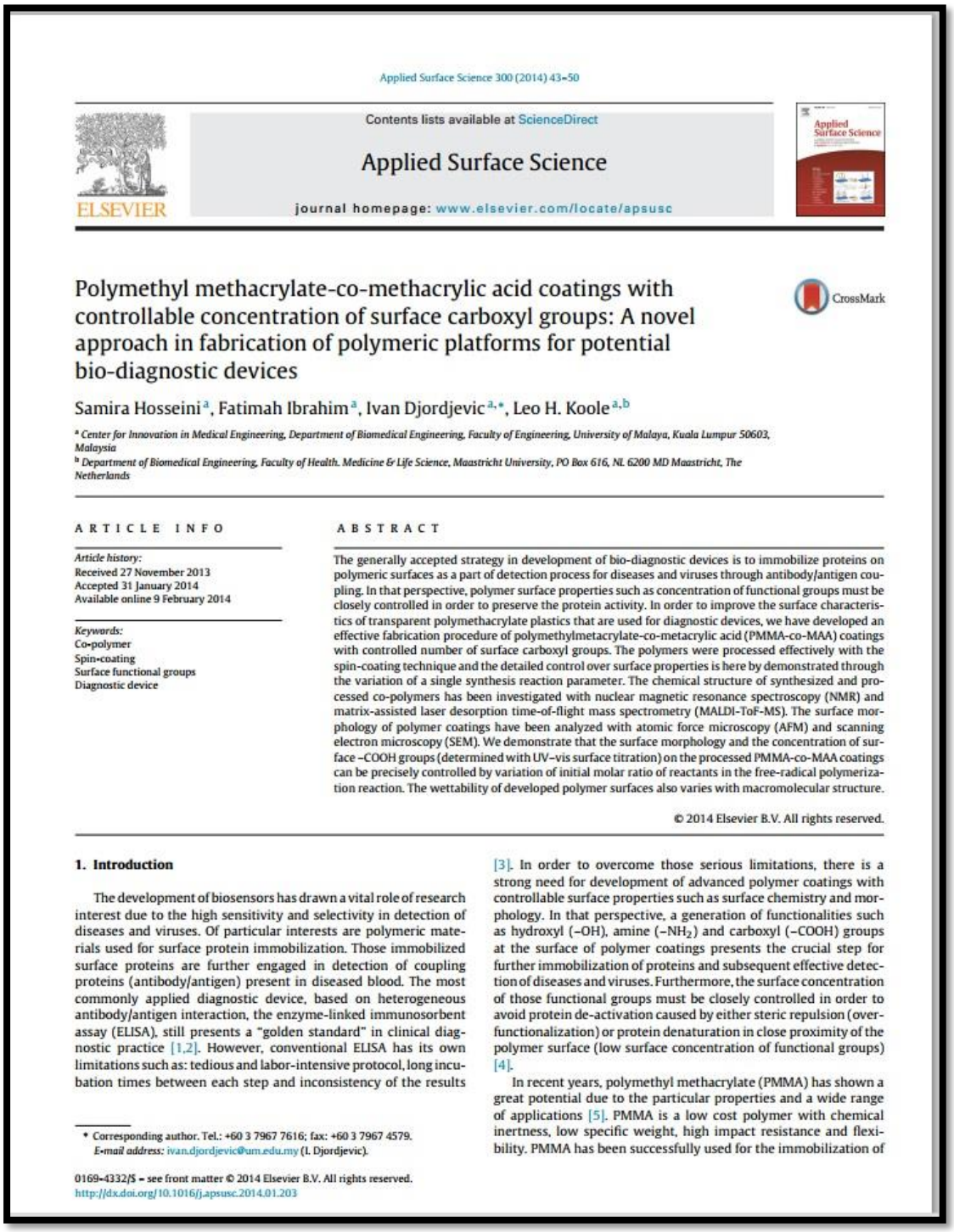
Research articles

- **Samira Hosseini**, Fatimah Ibrahim, Ivan Djordjevic and Leo. H. Koole . Polymethyl methacrylate-co-methacrylic acid coatings with controllable concentration of surface carboxyl groups: a novel approach in fabrication of polymeric platforms for potential bio-diagnostic devices. *Journal: Applied Surface Science, DOI: 10.1016/j.apsusc.2014.01.20.*



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Polymethyl methacrylate-co-methacrylic acid coatings with controllable concentration of surface carboxyl groups: A novel approach in fabrication of polymeric platforms for potential bio-diagnostic devices

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ABSTRACT

The generally accepted strategy in development of bio-diagnostic devices is to immobilize proteins on polymeric surfaces as a part of detection process for diseases and viruses through antibody/antigen coupling. In that perspective, polymer surface properties such as concentration of functional groups must be closely controlled in order to preserve the protein activity. In order to improve the surface characteristics of transparent polymethacrylate plastics that are used for diagnostic devices, we have developed an effective fabrication procedure of polymethylmetacrylate-co-metacrylic acid (PMMA-co-MAA) coatings with controlled number of surface carboxyl groups. The polymers were processed effectively with the spin-coating technique and the detailed control over surface properties is here by demonstrated through the variation of a single synthesis reaction parameter. The chemical structure of synthesized and processed co-polymers has been investigated with nuclear magnetic resonance spectroscopy (NMR) and matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-ToF-MS). The surface morphology of polymer coatings have been analyzed with atomic force microscopy (AFM) and scanning electron microscopy (SEM). We demonstrate that the surface morphology and the concentration of surface –COOH groups (determined with UV–vis surface titration) on the processed PMMA-co-MAA coatings can be precisely controlled by variation of initial molar ratio of reactants in the free-radical polymerization reaction. The wettability of developed polymer surfaces also varies with macromolecular structure.

1. Introduction

The development of biosensors has drawn a vital role of research interest due to the high sensitivity and selectivity in detection of diseases and viruses. Of particular interests are polymeric materials used for surface protein immobilization. Those immobilized surface proteins are further engaged in detection of coupling proteins (antibody/antigen) present in diseased blood. The most commonly applied diagnostic device, based on heterogeneous antibody/antigen interaction, the enzyme-linked immunosorbent assay (ELISA), still presents a "golden standard" in clinical diagnostic practice [1,2]. However, conventional ELISA has its own limitations such as: tedious and labor-intensive protocol, long incubation times between each step and inconsistency of the results [3]. In order to overcome those serious limitations, there is a strong need for development of advanced polymer coatings with controllable surface properties such as surface chemistry and morphology. In that perspective, a generation of functionalities such as hydroxyl (–OH), amine ($-NH_2$) and carboxyl (–COOH) groups at the surface of polymer coatings presents the crucial step for further immobilization of proteins and subsequent effective detection of diseases and viruses. Furthermore, the surface concentration of those functional groups must be closely controlled in order to avoid protein de-activation caused by either steric repulsion (over-functionalization) or protein denaturation in close proximity of the polymer surface (low surface concentration of functional groups) [4].

In recent years, polymethyl methacrylate (PMMA) has shown a great potential due to the particular properties and a wide range of applications [5]. PMMA is a low cost polymer with chemical inertness, low specific weight, high impact resistance and flexibility. PMMA has been successfully used for the immobilization of

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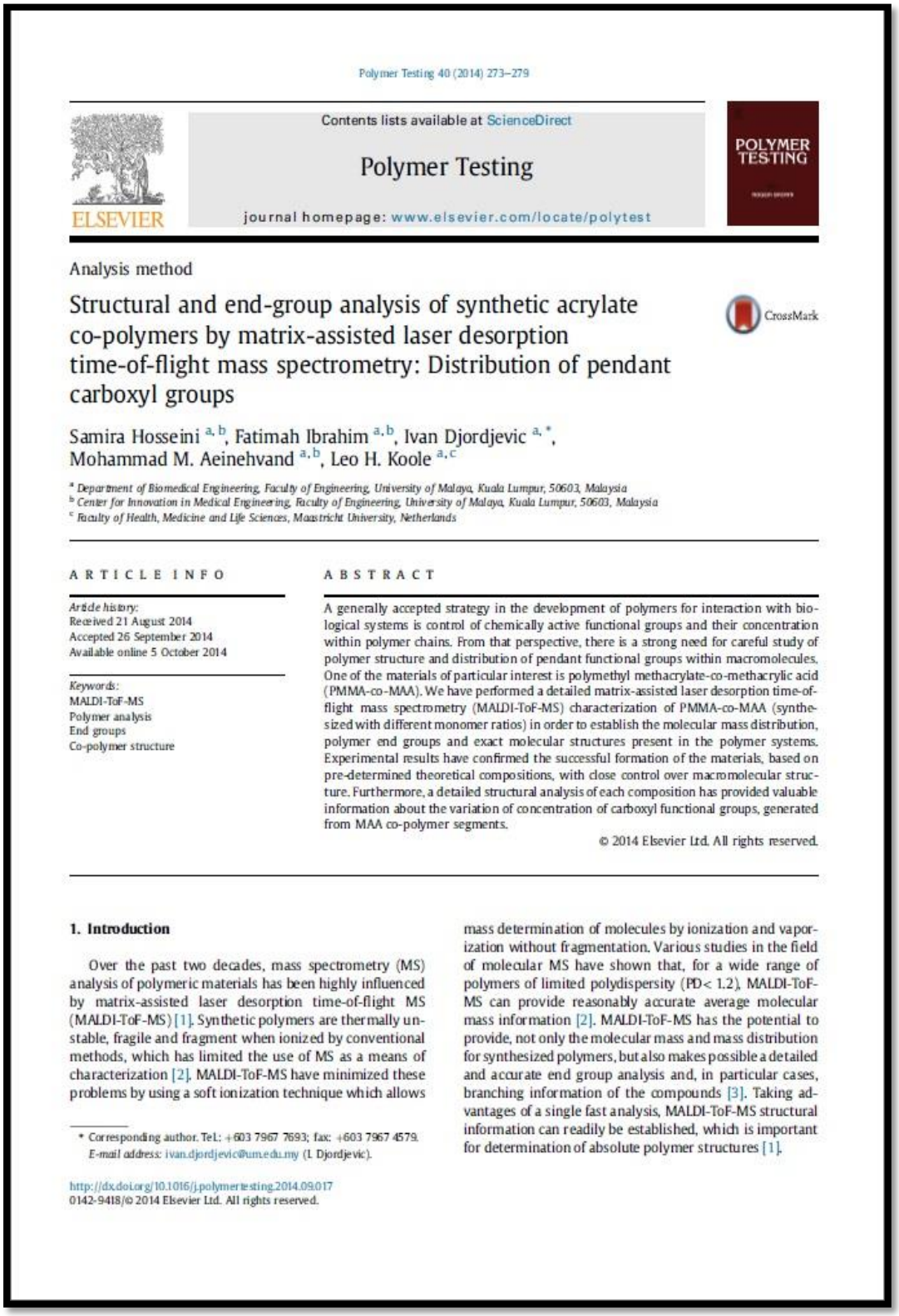
- **Samira Hosseini**, Fatimah Ibrahim, Ivan Djordjevic, Mohammad M. Aeinehvand, and Leo. H. Koole. Structural and end-group analysis of synthetic acrylate copolymers by matrix-assisted laser desorption time-of-flight mass spectrometry: distribution of pendant carboxyl groups. *Journal: Polymer Testing, DOI: 10.1016/j.polymertesting.2014.09.017.*



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Analysis method

Structural and end-group analysis of synthetic acrylate co-polymers by matrix-assisted laser desorption time-of-flight mass spectrometry: Distribution of pendant carboxyl groups

Samira Hosseini [a, b], Fatimah Ibrahim [a, b], Ivan Djordjevic [a, *], Mohammad M. Aeinehvand [a, b], Leo H. Koole [a, c]

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ABSTRACT

A generally accepted strategy in the development of polymers for interaction with biological systems is control of chemically active functional groups and their concentration within polymer chains. From that perspective, there is a strong need for careful study of polymer structure and distribution of pendant functional groups within macromolecules. One of the materials of particular interest is polymethyl methacrylate-co-methacrylic acid (PMMA-co-MAA). We have performed a detailed matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-ToF-MS) characterization of PMMA-co-MAA (synthesized with different monomer ratios) in order to establish the molecular mass distribution, polymer end groups and exact molecular structures present in the polymer systems. Experimental results have confirmed the successful formation of the materials, based on pre-determined theoretical compositions, with close control over macromolecular structure. Furthermore, a detailed structural analysis of each composition has provided valuable information about the variation of concentration of carboxyl functional groups, generated from MAA co-polymer segments.

1. Introduction

Over the past two decades, mass spectrometry (MS) analysis of polymeric materials has been highly influenced by matrix-assisted laser desorption time-of-flight MS (MALDI-ToF-MS) [1]. Synthetic polymers are thermally unstable, fragile and fragment when ionized by conventional methods, which has limited the use of MS as a means of characterization [2]. MALDI-ToF-MS have minimized these problems by using a soft ionization technique which allows mass determination of molecules by ionization and vaporization without fragmentation. Various studies in the field of molecular MS have shown that, for a wide range of polymers of limited polydispersity (PD< 1.2), MALDI-ToF-MS can provide reasonably accurate average molecular mass information [2]. MALDI-ToF-MS has the potential to provide, not only the molecular mass and mass distribution for synthesized polymers, but also makes possible a detailed and accurate end group analysis and, in particular cases, branching information of the compounds [3]. Taking advantages of a single fast analysis, MALDI-ToF-MS structural information can readily be established, which is important for determination of absolute polymer structures [1].

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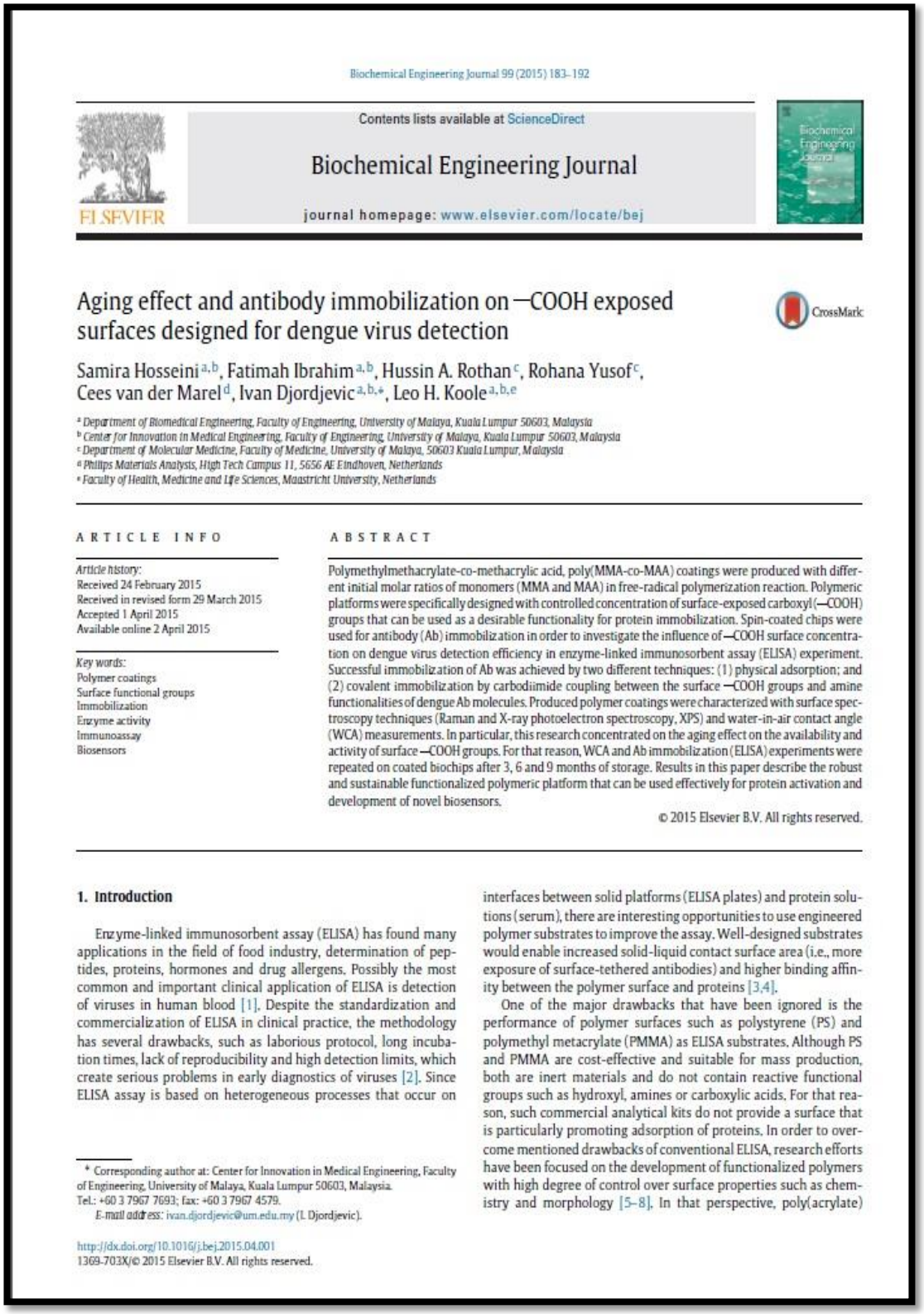

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Aging effect and antibody immobilization on —COOH exposed surfaces designed for dengue virus detection

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ABSTRACT

Polymethylmethacrylate-co-methacrylic acid, poly(MMA-co-MAA) coatings were produced with different initial molar ratios of monomers (MMA and MAA) in free-radical polymerization reaction. Polymeric platforms were specifically designed with controlled concentration of surface-exposed carboxyl (—COOH) groups that can be used as a desirable functionality for protein immobilization. Spin-coated chips were used for antibody (Ab) immobilization in order to investigate the influence of —COOH surface concentration on dengue virus detection efficiency in enzyme-linked immunosorbent assay (ELISA) experiment. Successful immobilization of Ab was achieved by two different techniques: (1) physical adsorption; and (2) covalent immobilization by carbodiimide coupling between the surface —COOH groups and amine functionalities of dengue Ab molecules. Produced polymer coatings were characterized with surface spectroscopy techniques (Raman and X-ray photoelectron spectroscopy, XPS) and water-in-air contact angle (WCA) measurements. In particular, this research concentrated on the aging effect on the availability and activity of surface —COOH groups. For that reason, WCA and Ab immobilization (ELISA) experiments were repeated on coated biochips after 3, 6 and 9 months of storage. Results in this paper describe the robust and sustainable functionalized polymeric platform that can be used effectively for protein activation and development of novel biosensors.

1. Introduction

Enzyme-linked immunosorbent assay (ELISA) has found many applications in the field of food industry, determination of peptides, proteins, hormones and drug allergens. Possibly the most common and important clinical application of ELISA is detection of viruses in human blood [1]. Despite the standardization and commercialization of ELISA in clinical practice, the methodology has several drawbacks, such as laborious protocol, long incubation times, lack of reproducibility and high detection limits, which create serious problems in early diagnostics of viruses [2]. Since ELISA assay is based on heterogeneous processes that occur on interfaces between solid platforms (ELISA plates) and protein solutions (serum), there are interesting opportunities to use engineered polymer substrates to improve the assay. Well-designed substrates would enable increased solid-liquid contact surface area (i.e., more exposure of surface-tethered antibodies) and higher binding affinity between the polymer surface and proteins [3,4].

One of the major drawbacks that have been ignored is the performance of polymer surfaces such as polystyrene (PS) and polymethyl metacrylate (PMMA) as ELISA substrates. Although PS and PMMA are cost-effective and suitable for mass production, both are inert materials and do not contain reactive functional groups such as hydroxyl, amines or carboxylic acids. For that reason, such commercial analytical kits do not provide a surface that is particularly promoting adsorption of proteins. In order to overcome mentioned drawbacks of conventional ELISA, research efforts have been focused on the development of functionalized polymers with high degree of control over surface properties such as chemistry and morphology [5–8]. In that perspective, poly(acrylate)

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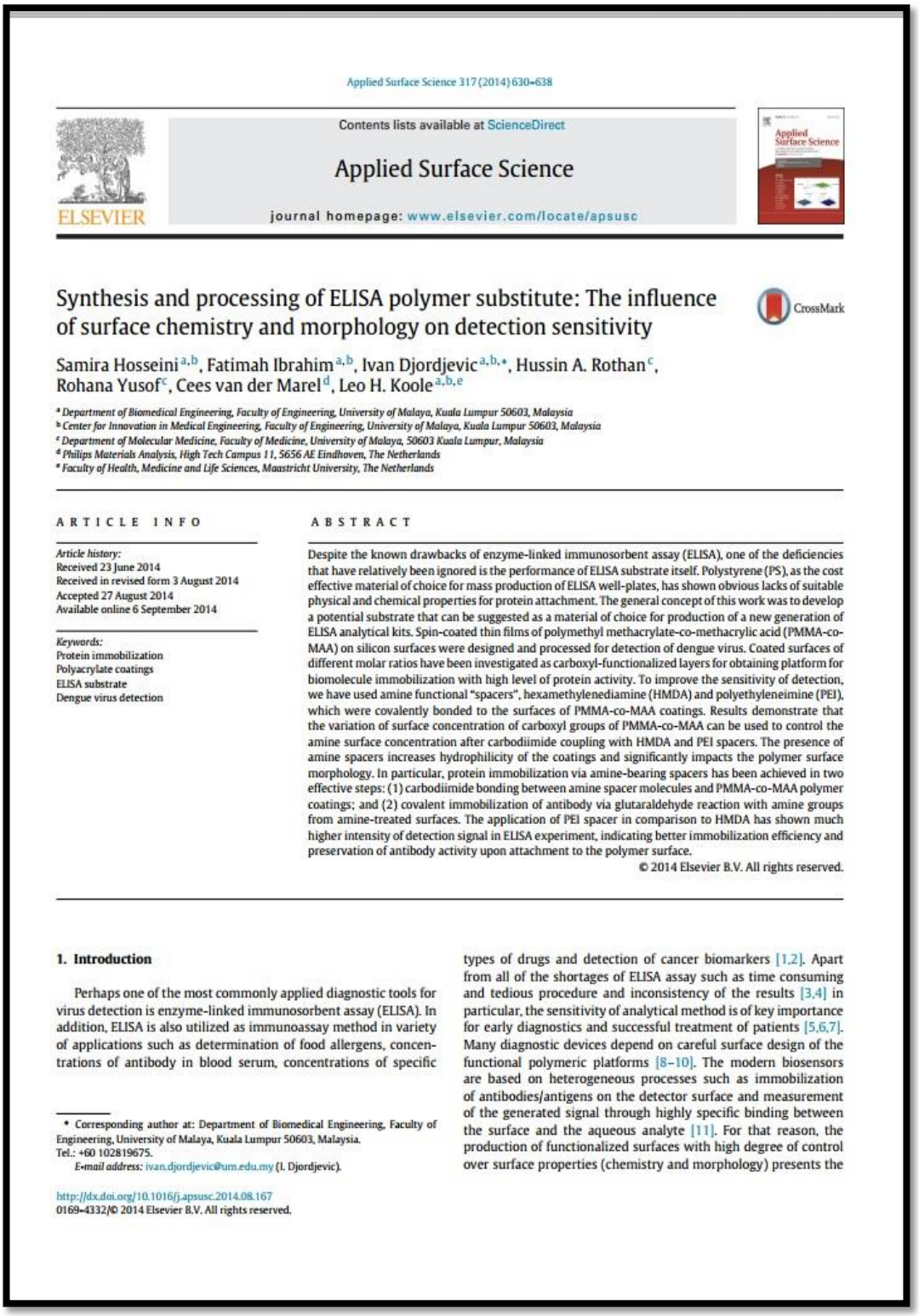

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Synthesis and processing of ELISA polymer substitute: The influence of surface chemistry and morphology on detection sensitivity

Samira Hosseini[a,b], Fatimah Ibrahim[a,b], Ivan Djordjevic[a,b,*], Hussin A. Rothan[c], Rohana Yusof[c], Cees van der Marel[d], Leo H. Koole[a,b,e]

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ABSTRACT

Despite the known drawbacks of enzyme-linked immunosorbent assay (ELISA), one of the deficiencies that have relatively been ignored is the performance of ELISA substrate itself. Polystyrene (PS), as the cost effective material of choice for mass production of ELISA well-plates, has shown obvious lacks of suitable physical and chemical properties for protein attachment. The general concept of this work was to develop a potential substrate that can be suggested as a material of choice for production of a new generation of ELISA analytical kits. Spin-coated thin films of polymethyl methacrylate-co-methacrylic acid (PMMA-co-MAA) on silicon surfaces were designed and processed for detection of dengue virus. Coated surfaces of different molar ratios have been investigated as carboxyl-functionalized layers for obtaining platform for biomolecule immobilization with high level of protein activity. To improve the sensitivity of detection, we have used amine functional "spacers", hexamethylenediamine (HMDA) and polyethyleneimine (PEI), which were covalently bonded to the surfaces of PMMA-co-MAA coatings. Results demonstrate that the variation of surface concentration of carboxyl groups of PMMA-co-MAA can be used to control the amine surface concentration after carbodiimide coupling with HMDA and PEI spacers. The presence of amine spacers increases hydrophilicity of the coatings and significantly impacts the polymer surface morphology. In particular, protein immobilization via amine-bearing spacers has been achieved in two effective steps: (1) carbodiimide bonding between amine spacer molecules and PMMA-co-MAA polymer coatings; and (2) covalent immobilization of antibody via glutaraldehyde reaction with amine groups from amine-treated surfaces. The application of PEI spacer in comparison to HMDA has shown much higher intensity of detection signal in ELISA experiment, indicating better immobilization efficiency and preservation of antibody activity upon attachment to the polymer surface.

1. Introduction

Perhaps one of the most commonly applied diagnostic tools for virus detection is enzyme-linked immunosorbent assay (ELISA). In addition, ELISA is also utilized as immunoassay method in variety of applications such as determination of food allergens, concentrations of antibody in blood serum, concentrations of specific types of drugs and detection of cancer biomarkers [1,2]. Apart from all of the shortages of ELISA assay such as time consuming and tedious procedure and inconsistency of the results [3,4] in particular, the sensitivity of analytical method is of key importance for early diagnostics and successful treatment of patients [5,6,7]. Many diagnostic devices depend on careful surface design of the functional polymeric platforms [8–10]. The modern biosensors are based on heterogeneous processes such as immobilization of antibodies/antigens on the detector surface and measurement of the generated signal through highly specific binding between the surface and the aqueous analyte [11]. For that reason, the production of functionalized surfaces with high degree of control over surface properties (chemistry and morphology) presents the

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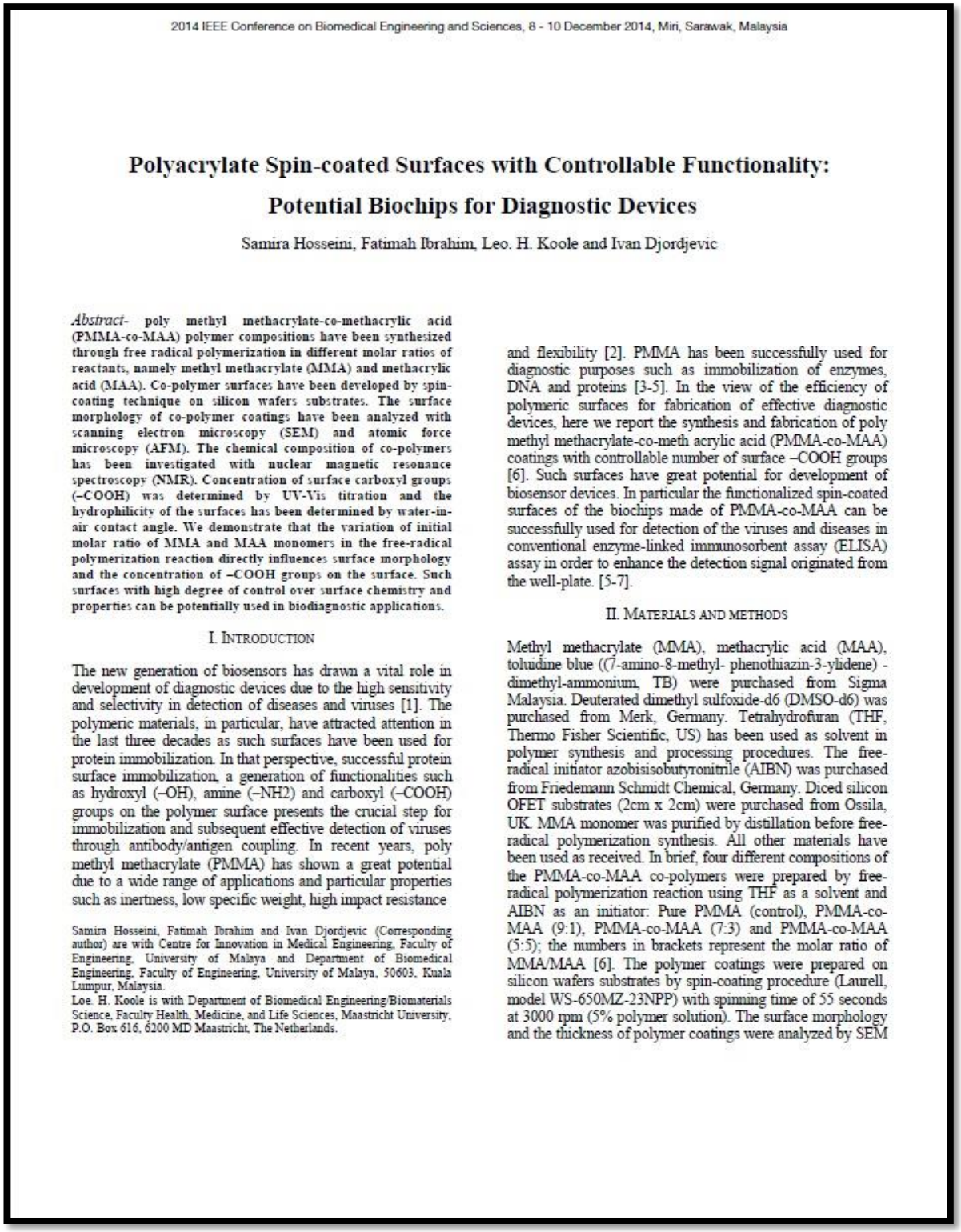
Conference proceedings

- **Samira Hosseini**, Fatimah Ibrahim, Leo. H. Koole and Ivan Djordjevic. Polyacrylate spin-coated surfaces with controllable functionality: potential biochips for diagnostic devices. *2014 IEEE Conference on Biomedical Engineering and Sciences, 8 - 10 December 2014, Miri, Sarawak, Malaysia*



2014 IEEE Conference on Biomedical Engineering and Sciences, 8 - 10 December 2014, Miri, Sarawak, Malaysia

Polyacrylate Spin-coated Surfaces with Controllable Functionality: Potential Biochips for Diagnostic Devices

Samira Hosseini, Fatimah Ibrahim, Leo. H. Koole and Ivan Djordjevic

Abstract- **poly methyl methacrylate-co-methacrylic acid (PMMA-co-MAA) polymer compositions have been synthesized through free radical polymerization in different molar ratios of reactants, namely methyl methacrylate (MMA) and methacrylic acid (MAA). Co-polymer surfaces have been developed by spin-coating technique on silicon wafers substrates. The surface morphology of co-polymer coatings have been analyzed with scanning electron microscopy (SEM) and atomic force microscopy (AFM). The chemical composition of co-polymers has been investigated with nuclear magnetic resonance spectroscopy (NMR). Concentration of surface carboxyl groups (–COOH) was determined by UV-Vis titration and the hydrophilicity of the surfaces has been determined by water-in-air contact angle. We demonstrate that the variation of initial molar ratio of MMA and MAA monomers in the free-radical polymerization reaction directly influences surface morphology and the concentration of –COOH groups on the surface. Such surfaces with high degree of control over surface chemistry and properties can be potentially used in biodiagnostic applications.**

I. Introduction

The new generation of biosensors has drawn a vital role in development of diagnostic devices due to the high sensitivity and selectivity in detection of diseases and viruses [1]. The polymeric materials, in particular, have attracted attention in the last three decades as such surfaces have been used for protein immobilization. In that perspective, successful protein surface immobilization, a generation of functionalities such as hydroxyl (–OH), amine (–NH2) and carboxyl (–COOH) groups on the polymer surface presents the crucial step for immobilization and subsequent effective detection of viruses through antibody/antigen coupling. In recent years, poly methyl methacrylate (PMMA) has shown a great potential due to a wide range of applications and particular properties such as inertness, low specific weight, high impact resistance and flexibility [2]. PMMA has been successfully used for diagnostic purposes such as immobilization of enzymes, DNA and proteins [3-5]. In the view of the efficiency of polymeric surfaces for fabrication of effective diagnostic devices, here we report the synthesis and fabrication of poly methyl methacrylate-co-meth acrylic acid (PMMA-co-MAA) coatings with controllable number of surface –COOH groups [6]. Such surfaces have great potential for development of biosensor devices. In particular the functionalized spin-coated surfaces of the biochips made of PMMA-co-MAA can be successfully used for detection of the viruses and diseases in conventional enzyme-linked immunosorbent assay (ELISA) assay in order to enhance the detection signal originated from the well-plate. [5-7].

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II. Materials and Methods

Methyl methacrylate (MMA), methacrylic acid (MAA), toluidine blue ((7-amino-8-methyl- phenothiazin-3-ylidene) - dimethyl-ammonium, TB) were purchased from Sigma Malaysia. Deuterated dimethyl sulfoxide-d6 (DMSO-d6) was purchased from Merk, Germany. Tetrahydrofuran (THF, Thermo Fisher Scientific, US) has been used as solvent in polymer synthesis and processing procedures. The free-radical initiator azobisisobutyronitrile (AIBN) was purchased from Friedemann Schmidt Chemical, Germany. Diced silicon OFET substrates (2cm x 2cm) were purchased from Ossila, UK. MMA monomer was purified by distillation before free-radical polymerization synthesis. All other materials have been used as received. In brief, four different compositions of the PMMA-co-MAA co-polymers were prepared by free-radical polymerization reaction using THF as a solvent and AIBN as an initiator: Pure PMMA (control), PMMA-co-MAA (9:1), PMMA-co-MAA (7:3) and PMMA-co-MAA (5:5); the numbers in brackets represent the molar ratio of MMA/MAA [6]. The polymer coatings were prepared on silicon wafers substrates by spin-coating procedure (Laurell, model WS-650MZ-23NPP) with spinning time of 55 seconds at 3000 rpm (5% polymer solution). The surface morphology and the thickness of polymer coatings were analyzed by SEM

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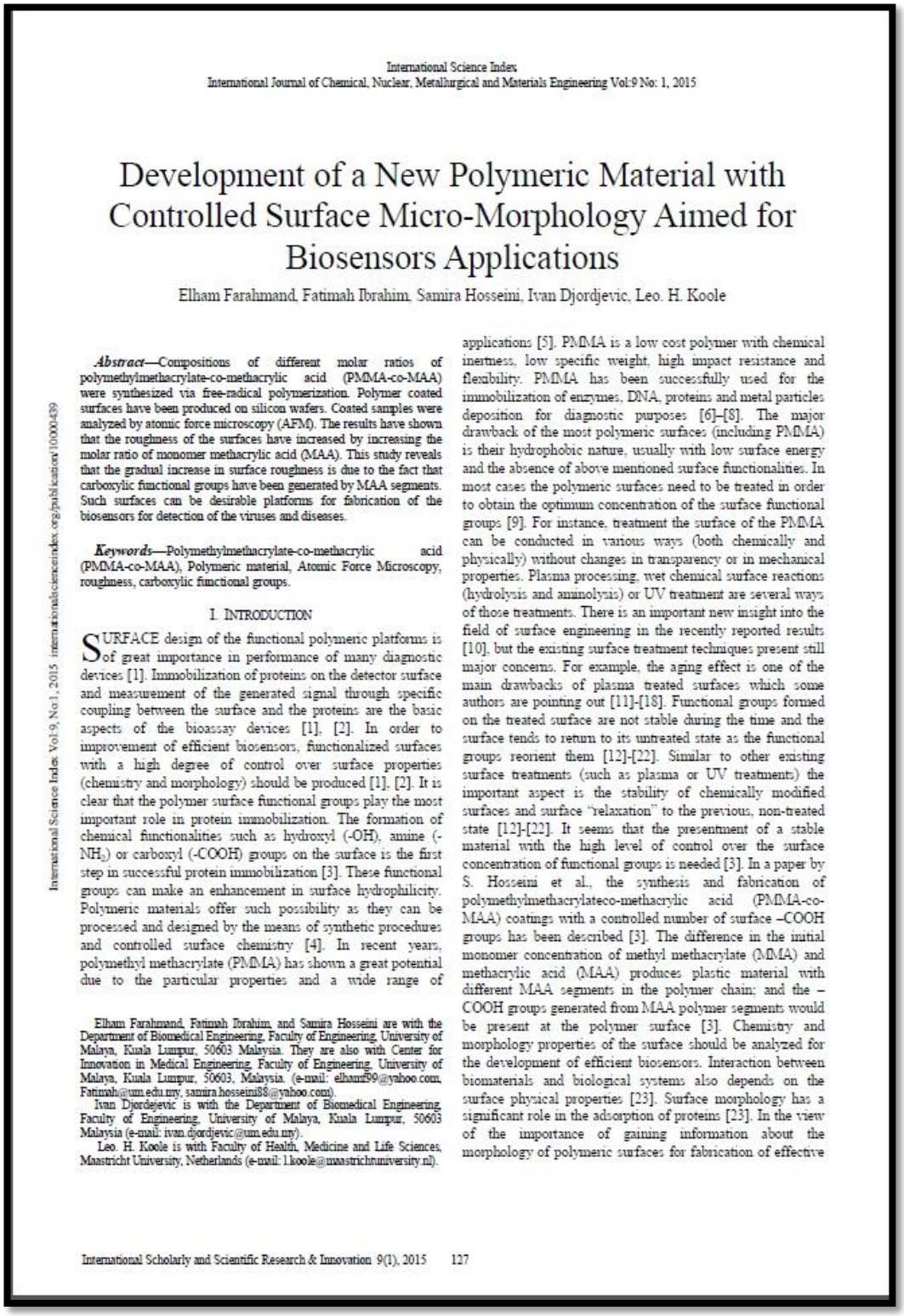

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Development of a New Polymeric Material with Controlled Surface Micro-Morphology Aimed for Biosensors Applications

Elham Farahmand, Fatimah Ibrahim, Samira Hosseini, Ivan Djordjevic, Leo. H. Koole

***Abstract*—**Compositions of different molar ratios of polymethylmethacrylate-co-methacrylic acid (PMMA-co-MAA) were synthesized via free-radical polymerization. Polymer coated surfaces have been produced on silicon wafers. Coated samples were analyzed by atomic force microscopy (AFM). The results have shown that the roughness of the surfaces have increased by increasing the molar ratio of monomer methacrylic acid (MAA). This study reveals that the gradual increase in surface roughness is due to the fact that carboxylic functional groups have been generated by MAA segments. Such surfaces can be desirable platforms for fabrication of the biosensors for detection of the viruses and diseases.

***Keywords*—**Polymethylmethacrylate-co-methacrylic acid (PMMA-co-MAA), Polymeric material, Atomic Force Microscopy, roughness, carboxylic functional groups.

I. Introduction

SURFACE design of the functional polymeric platforms is of great importance in performance of many diagnostic devices [1]. Immobilization of proteins on the detector surface and measurement of the generated signal through specific coupling between the surface and the proteins are the basic aspects of the bioassay devices [1], [2]. In order to improvement of efficient biosensors, functionalized surfaces with a high degree of control over surface properties (chemistry and morphology) should be produced [1], [2]. It is clear that the polymer surface functional groups play the most important role in protein immobilization. The formation of chemical functionalities such as hydroxyl (-OH), amine (-NH_2) or carboxyl (-COOH) groups on the surface is the first step in successful protein immobilization [3]. These functional groups can make an enhancement in surface hydrophilicity. Polymeric materials offer such possibility as they can be processed and designed by the means of synthetic procedures and controlled surface chemistry [4]. In recent years, polymethyl methacrylate (PMMA) has shown a great potential due to the particular properties and a wide range of applications [5]. PMMA is a low cost polymer with chemical inertness, low specific weight, high impact resistance and flexibility. PMMA has been successfully used for the immobilization of enzymes, DNA, proteins and metal particles deposition for diagnostic purposes [6]–[8]. The major drawback of the most polymeric surfaces (including PMMA) is their hydrophobic nature, usually with low surface energy and the absence of above mentioned surface functionalities. In most cases the polymeric surfaces need to be treated in order to obtain the optimum concentration of the surface functional groups [9]. For instance, treatment the surface of the PMMA can be conducted in various ways (both chemically and physically) without changes in transparency or in mechanical properties. Plasma processing, wet chemical surface reactions (hydrolysis and aminolysis) or UV treatment are several ways of those treatments. There is an important new insight into the field of surface engineering in the recently reported results [10], but the existing surface treatment techniques present still major concerns. For example, the aging effect is one of the main drawbacks of plasma treated surfaces which some authors are pointing out [11]-[18]. Functional groups formed on the treated surface are not stable during the time and the surface tends to return to its untreated state as the functional groups reorient them [12]-[22]. Similar to other existing surface treatments (such as plasma or UV treatments) the important aspect is the stability of chemically modified surfaces and surface "relaxation" to the previous, non-treated state [12]-[22]. It seems that the presentment of a stable material with the high level of control over the surface concentration of functional groups is needed [3]. In a paper by S. Hosseini et al., the synthesis and fabrication of polymethylmethacrylateco-methacrylic acid (PMMA-co-MAA) coatings with a controlled number of surface –COOH groups has been described [3]. The difference in the initial monomer concentration of methyl methacrylate (MMA) and methacrylic acid (MAA) produces plastic material with different MAA segments in the polymer chain; and the –COOH groups generated from MAA polymer segments would be present at the polymer surface [3]. Chemistry and morphology properties of the surface should be analyzed for the development of efficient biosensors. Interaction between biomaterials and biological systems also depends on the surface physical properties [23]. Surface morphology has a significant role in the adsorption of proteins [23]. In the view of the importance of gaining information about the morphology of polymeric surfaces for fabrication of effective

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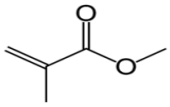
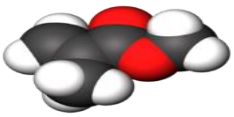
APPENDIX

1. Methyl methacrylate (MMA)

Methyl methacrylate is an organic compound (monomer) with known formula of $CH_2=C(CH_3)COOCH_3$ (Appendix Table 1). This colorless liquid can incorporate with the methyl ester groups of methacrylic acid (MAA) for production of PMMA-co-MAA via free radical polymerization reaction.

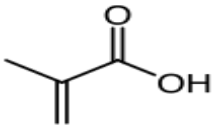
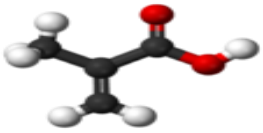
Appendix Table 1: Additional information for MMA.

Methyl methacrylate	
	
	
IUPAC name: methyl 2-methylpropenoate	
Other names: MMA, 2-(methoxycarbonyl)-1-propene	
Identifiers	
CAS number	80-62-6
Properties	
Molecular formula	$C_5H_8O_2$
Molar mass	100.12 g mol^{-1}
Appearance	Colorless liquid
Density	0.94 g/cm^3
Melting point	-48 °C, 225 K, -54 °F
Boiling point	101 °C, 374 K, 214 °F
Solubility in water	1.5 g/100 ml

2. Methacrylic acid (MAA)

Methacrylic acid (MAA) is an organic monomer (Appendix Table 2). This compound is colorless and viscous liquid with an acrid unpleasant odor. It is soluble in warm water and miscible with most organic solvents. The presence of carboxyl groups in the structure of monomer makes the compound desirable for surface engineering due to the presence of surface –COOH groups generated from MAA segments.

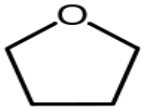
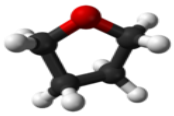
Appendix Table 2: Additional information for MAA.

Methacrylic acid	
	
	
IUPAC name: 2-methylpropenoic acid	
Other names: MAA, 2-methyl-2-propenoic acid	
Identifiers	
CAS number	79-41-4
Properties	
Molecular formula	$C_4H_6O_2$
Molar mass	86.06 g/mol
Density	1.015 g/cm^3
Melting point	14 - 15 °C
Boiling point	161 °C

3. Tetrahydrofuran (THF)

Tetrahydrofuran (THF) is an organic compound with the formula $(CH_2)_4O$ (Appendix Table 3). It is a water-miscible colorless organic liquid with low viscosity in the standard temperature and pressure. This heterocyclic compound is known as one of the most polar ethers with a wide liquid range, which is a useful solvent with variety of applications.

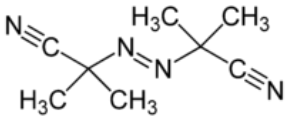
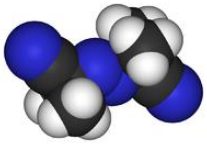
Appendix Table 3: Additional information for THF.

Tetrahydrofuran	
	
	
IUPAC name: Oxolane	
Other names: THF, tetrahydrofuran, 1,4-epoxybutane, butylene oxide, cyclotetramethylene oxide, oxacyclopentane	
Identifiers	
CAS number	109-99-9
Properties	
Molecular formula	C_4H_8O
Molar mass	72.11 g mol^{-1}
Density	0.8892 g/cm^3 @ 20 °C, liquid
Melting point	−108.4 °C, 165 K, -163 °F
Boiling point	66 °C, 339 K, 151 °F

4. Azobisisobutyronitrile (AIBN)

Azobisisobutyronitrile (AIBN) is an organic compound with the formula $[(CH_3)_2C(CN)]_2N_2$ (Appendix Table 4). This white powder is soluble inside alcohols and other common organic solvents but not in water. It is often used in polymerization reactions as a radical initiator.

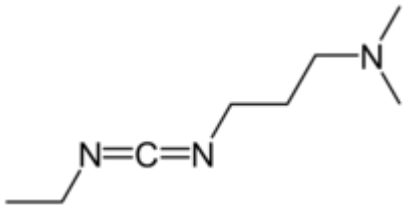
Appendix Table 4: Additional information for AIBN.

Azobisisobutyronitrile	
	
	
IUPAC name: 2,2′-Azobis(2-methylpropionitrile), 2-(azo(1-cyano-1-methylethyl))-2-methylpropanenitrile	
Other names: Azobisisobutyronitrile, Azobisisobutylonitrile, AIBN	
Identifiers	
2CAS number	78-67-1
Properties	
Molecular formula	$C_8H_{12}N_4$
Molar mass	164.21 g/mol
Density	1.1 g cm^{-3}
Melting point	103-105 °C, 376-378 K, 217-221 °F

5. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC)

1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) is a water soluble carbodiimide agent (Appendix Table 5). It is typically employed in the 4.0-6.0 pH range. General use of this compound is for activation of carboxyl groups for coupling of primary amines to yield amide bonds. EDC is often used in combination with N-hydroxysuccinimide (NHS) or sulfo-NHS to increase coupling efficiency by creating a more reactive amine product.

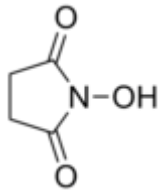
Appendix Table 5: Additional information for EDC.

1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide	
	
IUPAC name: 3-(Ethyliminomethyleneamino)-*N*,*N*-dimethylpropan-1-amine	
Identifiers	
CAS number	1892-57-5
Properties	
Molecular formula	$C_8H_{17}N_3$
Molar mass	155.24 g mol^{-1}

6. N-Hydroxysuccinimide (NHS)

N-Hydroxysuccinimide (NHS) is an organic compound with the known formula of $C_4H_5NO_3$ (Appendix Table 6). NHS is slightly acidic, irritant to body, in particular to skin and eyes. NHS is commonly applied in biochemistry where it can be used as an activating reagent for converting carboxylic acids to intermediate amides groups for further coupling with proteins while a normal carboxylic acid would just form a salt with an amine.

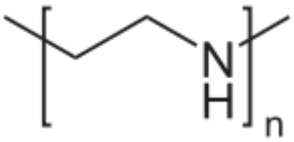
Appendix Table 6: Additional information for NHS.

***N*-Hydroxysuccinimide**	
	
IUPAC name: 1-Hydroxy-2,5-pyrrolidinedione	
Other names: 1-hydroxypyrrolidine-2,5-dione,HOSu	
Identifiers	
CAS number	6066-82-6
Properties	
Molecular formula	$C_4H_5NO_3$
Molar mass	115.09 g/mol
Appearance	Colorless solid
Melting point	95 °C, 368 K, 203 °F

7. Polyethylenimine (PEI)

Polyethylenimine (PEI) is a polymer compound in two forms of linear and branched structures (Appendix Table 7). Linear polyethyleneimine contains all secondary amines. However, branched PEI contains primary, secondary and tertiary amino groups (Yemul & Imae, 2008). Branched PEI can be polymerized by using ring opening reaction. Different degree of branching can be achieved by controlling the reaction parameters. PEI is known as a commonly used amine-bearing spacer for efficient protein immobilization (Bai et al., 2006).

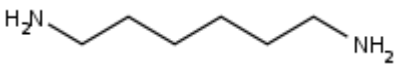
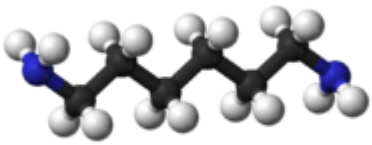
Appendix Table 7: Additional information for PEI.

Polyethylenimine	
	
IUPAC name: Poly (iminoethylene)	
Other names: Polyaziridine, Poly[imino(1,2-ethanediyl)]	
Identifiers	
CAS number	9002-98-6
Properties	
Molecular formula	$(C_2H_5N)_n$, linear form
Molar mass	variable

8. Hexamethylenediamine (HMDA)

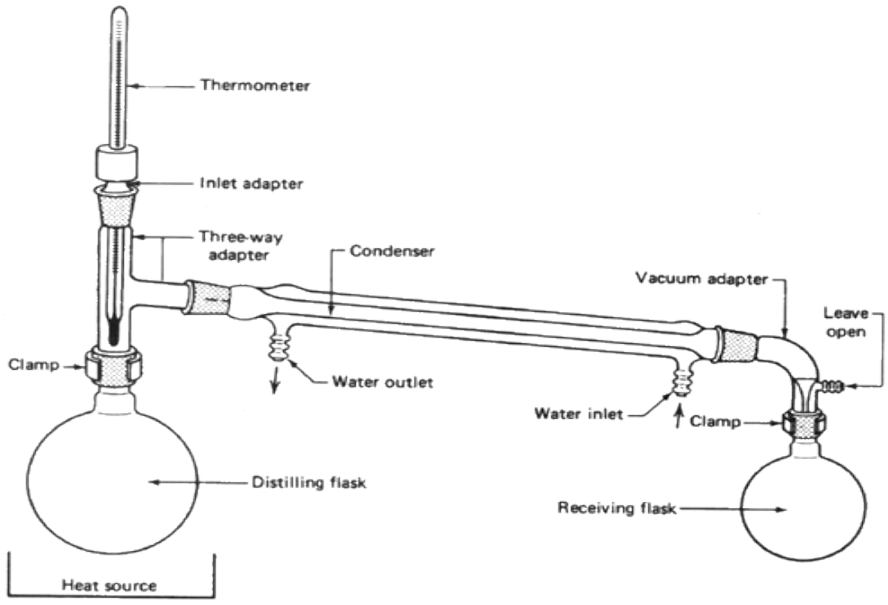
Hexamethylenediamine (HMDA) is an organic compound with the formula of $H_2N(CH_2)_6NH_2$ (Appendix Table 8). HMDA is a diamine molecule including a chain of hexamethylene terminated with amine functional groups. This colorless solid compound has a strong amine odor. HMDA has application in biomolecular interaction as an amine-bearing spacer.

Appendix Table 8: Additional information for HMDA.

Hexamethylenediamine	
	
	
IUPAC name: Hexane-1,6-diamine	
Other names: 1,6-Diaminohexane, 1,6-Hexanediamine	
Identifiers	
CAS number	124-09-4
Properties	
Molecular formula	$C_6H_{16}N_2$
Molar mass	116.20 g mol^{-1}
Appearance	Colorless crystals
Density	0.84 g/mL
Melting point	39-42 °C, 312-315 K, 102-108 °F
Boiling point	205 °C, 477.7 K, 400 °F
Solubility in water	490 g L^{-1}

9. Monomer distillation procedure

Distillation is a method of separating mixtures based on differences in volatility of components in a boiling liquid mixture. The simple distillation setup is shown in Appendix Figure 1. Several steps were followed for purification of MMA (monomer), in first step some glass beads were added to the monomer inside the distillation flask. By using the heating mantle, the temperature was controlled up to the boiling point of the monomer (101 °C). Condensed drops gradually appeared in receiving flask. Distilled monomer was kept in the fridge and stored for further experiments.

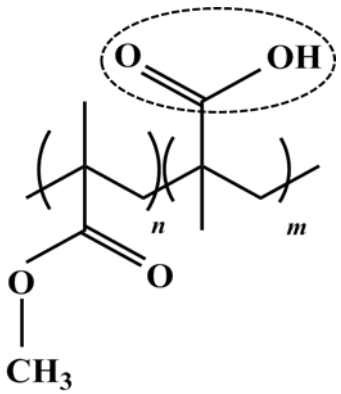


Appendix Figure 1: Distillation set up for purification of the MMA monomer prior to polymerization reaction.

10. Synthesis of PMMA-co-MAA

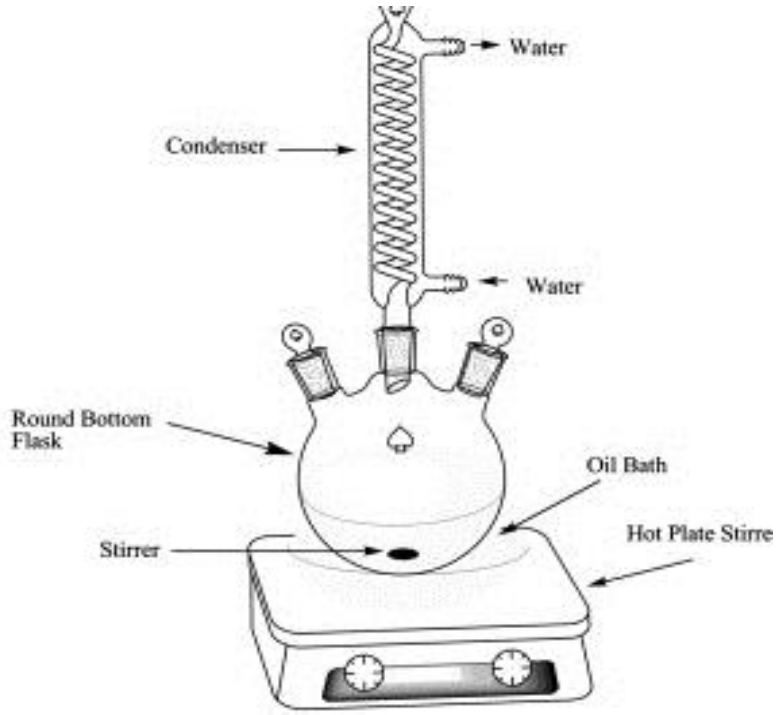
Four different compositions of the PMMA-co-MAA copolymers were prepared by free radical polymerization reaction by using THF as a solvent and AIBN as an initiator. The abbreviations of the copolymers have been used identifying the initial molar ratios of the monomers. For instance, PMMA-co-MAA (9:1) corresponds to 90% of MMA and 10% of MAA in reaction mixture, PMMA-co-MAA (7:3), contains 70% MMA and

30% MAA, and PMMA-co-MAA (5:5) has equal percentage from both monomers, 50% (Appendix Figure 2).



Appendix Figure 2: Proposed synthesis and chemical structure of copolymer PMMA-co-MAA.

The pure PMMA (polymerization of MMA with AIBN initiator) was synthesized in the same reaction conditions and used as control in all experiments. A three-neck round-bottom flask was fitted with a condenser and sealed inlet used for reactants feed (Appendix Figure 3). The set up was charged with 50 ml of THF and pre-calculated amount of MMA and stirred for 5 minutes. A mixture of MAA and AIBN (0.328g) was gradually added to the solution. Reaction was allowed to proceed for 6 hours at 90 ºC. At the final step, reaction mixture was poured into 1000 ml of distilled water and immediate formation of white precipitates confirmed successful polymerization. Mentioned precipitations were filtered and washed thoroughly with distilled water by using centrifuge. Freeze-drying has been used to remove the residual water and then samples were stored in the fridge before dissolution and subsequent chemical analysis (Hosseini, et al., 2014a).

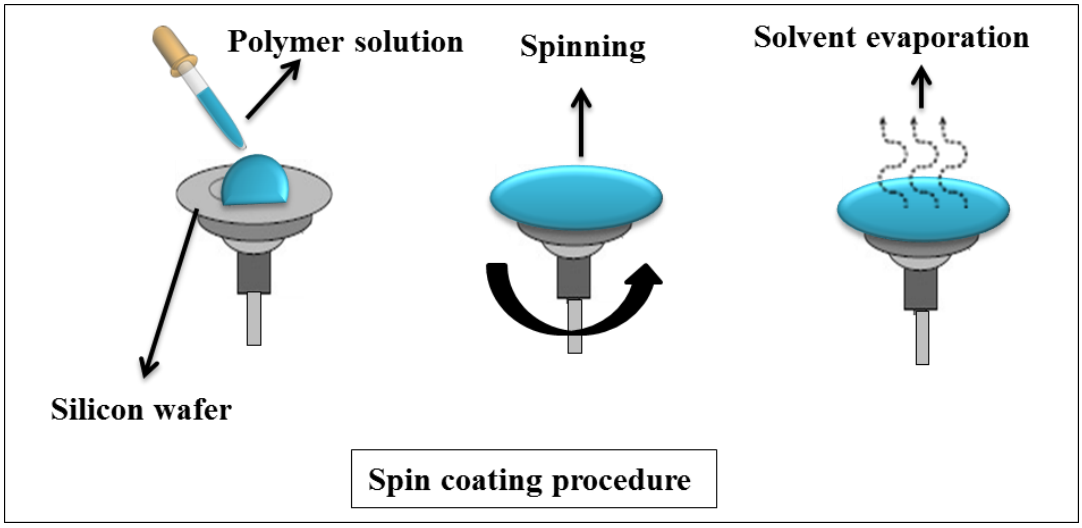


Appendix Figure 3: Polymerization setup for syntheses of PMMA-co-MAA in different molar ratios.

11. Spin-coating procedure

The polymer coatings were prepared on silicon wafers (as substrate) by spin-coating procedure. Silicon wafers were previously cleaned in three following protocol: (1) substrates were first immersed for 5 minutes in the solution of H_2O:H_2O_2:HCl with the ratio of 6:1:1; (2) secondly, substrates were taken out and immersed for another 5 minutes in solution of NH_4OH:H_2O:$H2O_2$ with the ratio of 1:5:1; (3) the final step has been completed by rinsing the substrates in the solution of H_2O:HF with the ratio of 1:1 for 5 minutes. Silicon wafers were then rinsed thoroughly and sonicated in purified water for 5 minutes between each washing step. After washing, substrates were sonicated again and dried under stream of nitrogen. After the cleaning, silicon wafers were coated using spin-coater (model WS-650MZ- 23NPP) with spinning time of 55 seconds in a single step at 3000 rpm (Appendix Figure 4). Coated samples were placed in oven (60 °C) for solvent evaporation overnight. 5% polymer solutions (THF) were

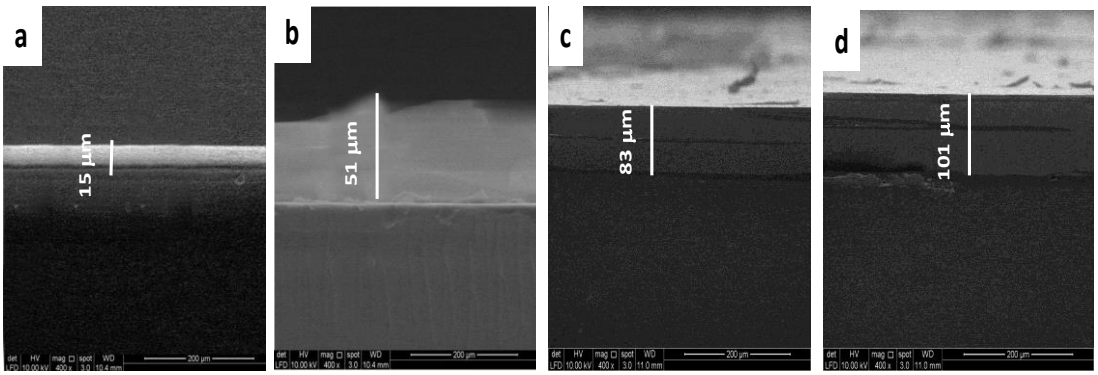
used for all copolymer compositions including the PMMA control (Hosseiniet al,. 2014a).



Appendix Figure 4: Spin-coating procedure.

12. SEM cross-section images of the HMDA treated biochips

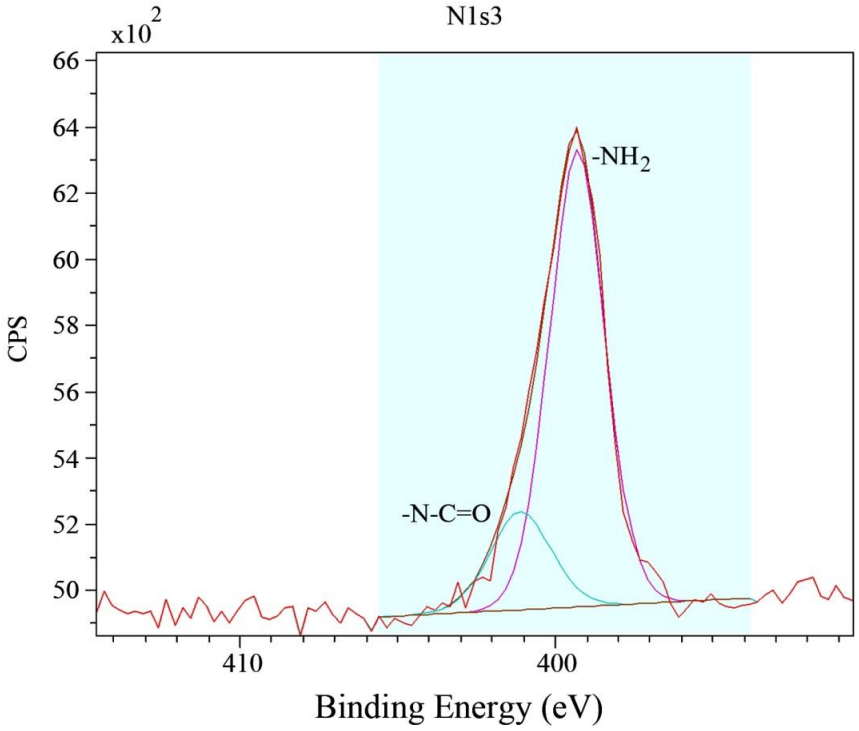
Cross section images of the aminated biochips can be seen in Appendix Figure 5. The coated surfaces of the biochips have been incubated in PBS solution for 1 hour prior to imaging. Samples have been washed by distilled water before analysis.



Appendix Figure 5: SEM cross section images of PEI treated coatings (after 1 hour incubation in PBS buffer): (a) PMMA-PEI; (b) PMMA-co-MAA-(9:1)-PEI; (c) PMMA-co-MAA-(7:3)-PEI; (d) PMMA-co-MAA-(5:5)-PEI.

13. XPS analysis of the HMDA treated biochips

The detected N1s peak of HMDA treated surfaces of the PMMA-co-MAA chips can be seen in Appendix Figure 6.

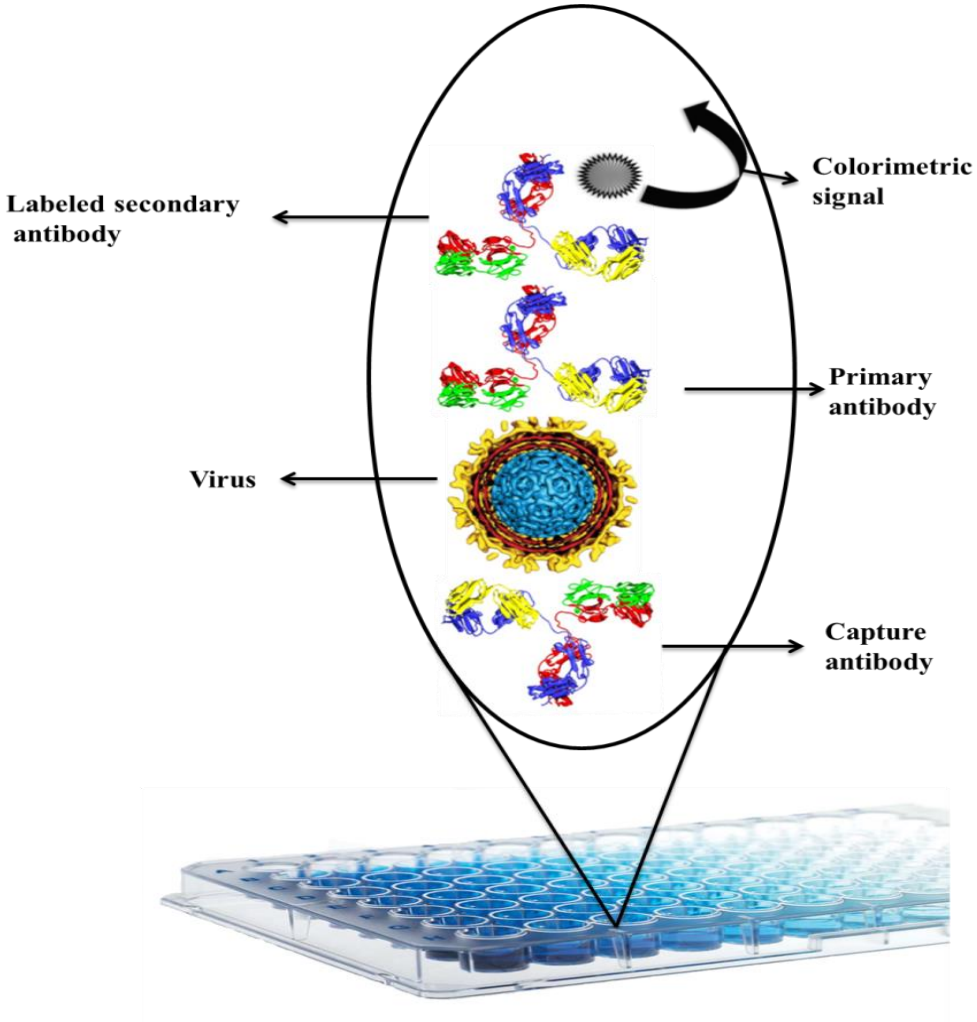


Appendix Figure 6: N1s peak of PMMA-co-MAA-HMDA showing two peaks assigned to amide (–N–C=O) and amine ($–NH_2$) groups.

14. Sandwich ELISA colorimetric assay

Invented in 1971 at Stockholm University (Sweden), ELISA rapidly found its position as one of the most well-known and widely applied analytical assays. ELISA has found variety of applications in many different areas such as food industry, toxicology, immunology, biological pharmacy and diagnostics. This analytical tool was frequently used for determination of food allergens, recognition of the cancer biomarkers, screening certain classes of drugs and detection of viruses. Among different protocols for performing ELISA such as direct, indirect, sandwich and competitive, sandwich ELISA has proven to be the most specific and reliable method for protein recognition. In this strategy, analyte of interest is locally immobilized between two antibodies from

both sides (Appendix Figure 8). Capture and primary antibodies that have sandwiched the virus between themselves are generated from different hosts thus incapable of binding one to another. Primary antibody (Ab), however, can further bind to the secondary, labeled Ab that generates fluorescence light emission (or color change) upon targeted protein recognition. The intensity of detection signal is proportional to the concentration of the virus.



Appendix Figure 8: General principle for sandwich ELISA.

In order to avoid uncertainties and minimize risk of non-specific binding, sandwich ELISA protocol has been selected as the protocol of choice in this study. Detailed procedure of sandwich ELISA can be found as follows:

14.1. Coating by capture antibody

Goat IgG anti Dengue Virus 2 (D-15): SC-325014 was diluted (1:300) in coating buffer (0.85 g of NaCl, 0.14 g of Na_2HPO_4 and 0.02g of NaH_2PO_4 in 100 ml of PBS) and used in sandwich ELISA assay. Each well of the ELISA well-plate was charged with 100µl of capture antibody and the incubation was carried on for 2 hour in 37°C.

14.2. Washing step

ELISA well-plates (both empty and with polymer coated silicon chips) were thoroughly washed with washing buffer (0.05% Tween 20 in PBS) prior to 15 minutes of saturation at room temperature with 200 µl per well of washing buffer. The washing steps were performed (3times×5 minutes) using shaker (1000 rpm). The same washing procedure has been performed between each two steps of the ELISA experiment.

14.3. Blocking step

Blocking buffer was prepared by adding 1 g of BSA to 100 ml of washing buffer. Each well was charged with 100 µl of blocking buffer in order to block the non-specific binding sites and achieve higher selectivity of the detection. Blocking step was performed in the incubator at 37°C for 1 hour.

14.4. Coating by virus

The enveloped Dengue virus has been diluted by using serial dilution and the representative concentration has been added to the wells. The overnight incubation in 4°C was done by adding 100 µl of the virus solution into each well.

14.5. Coating by primary antibody

Mouse IgG anti Dengue virus ICL2: SC-65725 was diluted (1:300) in diluting buffer (0.4g BSA, 4ml PBS buffer and 120µl of Trintonx-100 in 36 ml of distilled water).

Each well was charged by 100 µl of antibody solution and well-plates were placed in incubator of 37°C for 2 hour.

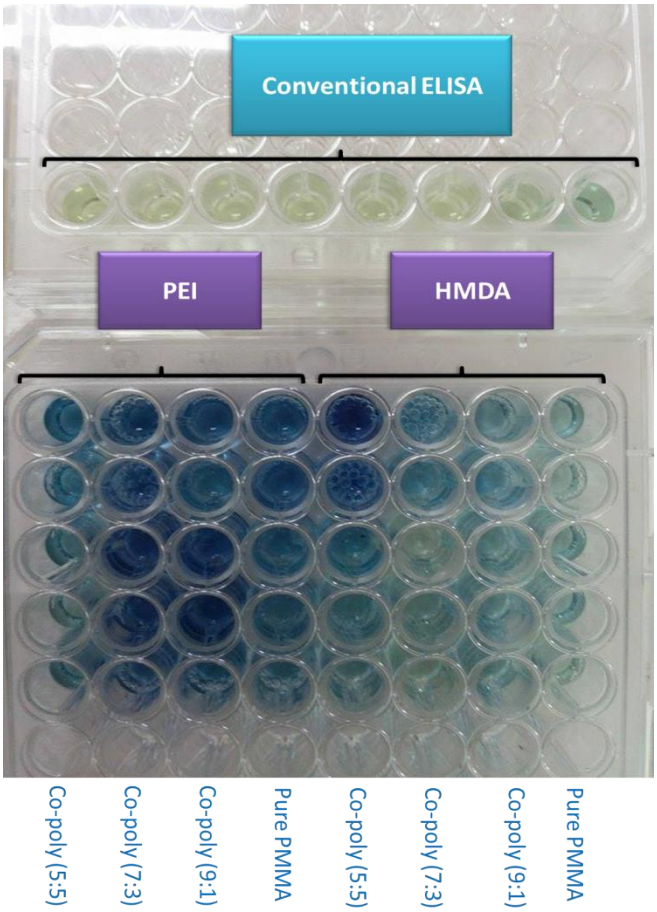
14.6. Coating by secondary antibody

In order to have a proper judgment of the performance of the coated surfaces, chips have been taken out from the well-plates and were placed in the new well-plates before further incubation. The secondary antibody (anti-mouse IgG (Fc specific)) has also been diluted in diluting buffer with the ratio of 1:200. ELISA well-plate were incubated in 100 µl of the secondary antibody solution, however the incubation was done just for 30 minutes.

14.7. Stopping Step

ELISA well-plates were thoroughly washed and charged by 100 µl of mixed substrate (Alkaline phosphatase blue microwell substrate components A and B). After 10-15 minutes the reaction has been stopped by using 50 µl of alkaline phosphatase stop solution (A585, Sigma) and the results were recorded by Bio-Rad (model680) in 570 nm. Negative controls were measured when the reaction was carried out in the absence of antigen.

Appendix Figure 7 presents the resulted calorimetric detection of the Dengue virus by using spacers of two sizes (HMDA and PEI). The difference in color intensity between conventional ELISA and biochips can be seen in this Figure.



Appendix Figure 7: Sandwich ELISA colorimetric results after detection of Dengue virus.